Identification and Comparative Analysis of Novel Factors from the Venom Gland of the Coastal Taipan (*Oxyuranus scutellatus*) and Related Species

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List of Keywords

Coastal taipan (*Oxyuranus scutellatus*), Australian elapid snake, Venom, Gene cloning, Recombinant protein expression, Toxin, Prothrombin activator, Phospholipase A$_2$, Pseudechetoxin, Neurotoxin, Natriuretic peptide, Calglandulin, L-amino acid oxidase.

Abstract

Snake venoms are a complex mixture of polypeptide and other molecules that adversely affect multiple homeostatic systems within their prey in a highly specific and targeted manner. Amongst the most potently toxic venoms in the world are those of the Australian venomous snakes, which belong almost exclusively to the elapid family. Their venoms posses a number of unique properties by which they target the mammalian cardiovascular and neuromuscular systems and are the focus for the identification of novel pharmacologically interesting compounds which may be of diagnostic or therapeutic benefit. Although much is known about the biochemical properties of Australia snake venoms as a whole, little research attention has focused upon individual components at the molecular level. This thesis describes the cloning, characterisation and comparative analysis of a number of unique toxins from the venom gland of the coastal taipan (*Oxyuranus scutellatus*) and a total of seven other related Australian snakes. These include the factor X- and factor V-like components of a prothrombin activator that causes a highly coagulable state in mammals. Comparative analysis of the sequences identified in this study, along with recombinant expression of an active form of the factor X-like component, provides important information on the structural, functional and evolutionary relationships of these molecules. Numerous other toxins were similarly identified and characterised including a pseudechetoxin-like protein, multiple phospholipase A_2 enzymes and neurotoxin isoforms as well as vasoactive venom natriuretic peptides. Identified transcripts included not only toxin sequences but also other cellular peptides implicated in toxin processing, including a calglandulin-like protein. This thesis is the first description of the majority of these molecules at either the cDNA or protein level, and provides a means to study the activity of individual components from snake venoms and probe their function within the systems they specifically target. This study represents the most detailed and comprehensive description to date of the cloning and characterisation of different genes associated with envenomation from Australian snakes.

Contents

List of Figures and Tables

List of Resulting Publications

St Pierre L., Masci PP., Filippovich I., Sorokina N., Marsh N., Miller DJ. and Lavin MF. (2005). Comparative analysis of prothrombin activators from the venom of Australian elapids. Mol Biol Evol. **22** (9): 1853-1864.

Filippovich I., Sorokina N., St Pierre L., Flight S., de Jersey J., Perry N., Masci PP. and Lavin MF. (2005). Cloning and functional expression of venom prothrombin activator protease from *Pseudonaja textilis* with whole blood procoagulant activity. Br J Haem. **131**: 237-246.

St Pierre L., Woods R., Earl S., Masci PP. and Lavin MF. (2005). Identification and analysis of venom gland-specific genes from the coastal taipan (*Oxyuranus scutellatus*) and related species. Cell Mol Life Sci. Nov 2.

St Pierre L., Flight S., Masci PP., de Jersey J. and Lavin MF. (2005). Cloning and characterisation of vasoactive natriuretic peptides from the venom of Australian snakes. (In preparation).

St Pierre L., Fisher H., Masci PP., Adams D. and Lavin MF. (2005). Cloning and characterisation of multiple neurotoxin isoforms from the venom of Australian elapids. (In preparation).

Earl S., St Pierre L., Birrell G., Wallis T., Masci PP., Gorman J., de Jersey J. and Lavin MF. (2005). Identification and cDNA cloning of venom nerve growth factor from the Australian elapid snakes. (Submitted).

List of Commonly Used Abbreviations

ACE……………………….Angiotensin Converting Enzyme

CALGP……………………...Calglandulin-like Protein

CNG ion channel……………Cyclic Nucleotide Gated ion channel

CRISP……………………….Cysteine-rich secretory protein

DIC………………………….Disseminted Intravascular Coagulation

Factor Xa……………………Activated Factor Ten

Factor Va……………………Activated Factor Five

LAAO……………………….L-amino acid oxidase

LD50………………………...Lethal Dose 50

LNTX……………………….Long Chain Neurotoxin

nAChR……………..………..Nicotinic Acetylcholine Receptor

OD…………………………...Optical Density

PCR…………...…….………Polymerase Chain Reaction

PLA_2………………………...Phospholipase A_2

RACE……………………….Rapid Amplification of cDNA Ends

RVV………………………….Russell's Viper Venom

SNTX…………...…….….…..Short Chain Neurotoxin

UTR…………………………Untranslated Region

Statement of Original Authorship

The work contained in this thesis has not been previously submitted for a degree or diploma at another higher education institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person except where due reference is made.

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CHAPTER 1
General Introduction

Snake venoms are a complex mixture of pharmacologically active protein and polypeptide toxins that have specifically evolved to alter the physiological mechanisms of their prey. Their components are typically small in size, cysteine rich and often exert a multitude of functions with specific molecular targets, including ion channels and a range of enzymes (Menez, 1998). The majority of venomous snakes belong to one of two taxonomic families, the *Elapidae*, including cobras, kraits, mambas, coral snakes and Australian elapids or the *Viperidae*, which include pit vipers (rattlesnakes, copperheads and cottonmouths) and true vipers (European viper, gaboon viper and puff adder) (Matsui *et al.*, 2000). However it is the Australian venomous snakes, which belong almost solely to the elapid family, that are recognised as having amongst the most potently toxic venom in the world (Broad *et al.*, 1979).

The venom from a single Australian elapid snake may contain upwards of a hundred different proteins. These toxins have previously been clustered into three principle groups, including the phospholipases, prothrombin activating enzymes and peptidic neurotoxins (Fry, 1999). However there has been recent evidence to suggest that the composition of these venoms is not solely limited to these three subgroups. For example, recent studies have identified the presence of low molecular weight natriuretic peptides in the venom of members of the *Oxyuranus* genus thought to play a role in the vasoactive nature of the venom, whilst high molecular weight L-amino acid oxidase-like proteins that demonstrate potent antibacterial effects have also been observed in the venom of the mulga, *Pseudechis australis* (Fry *et al.*, 2005; Stiles *et al.*, 1991). A detailed review of all the currently identified components of Australian elapid venoms will be provided later in this chapter. Snake venoms also contain a small component (less than 10%) of non-polypeptide constituents including amines, carbohydrates, nucleosides, lipids and metal ions, but on the whole these are the least biologically active elements.

The amount of venom that a snake produces can obviously affect the severity of envenomation following a bite. Determinants of the quantity of venom delivered by an individual snake at a bite site include a range of factors such as the body and head size, sex, diet, geographic location and species of snake (Mirtschin *et al.*, 2002; Morrison *et al.*, 1983).

These factors are also implicated in the variation within species of the components identified in the venom and their relative activities (Williams and White, 1992). Evidence suggests however, that there is no significant age-dependent difference in the composition of the venom between juvenile and adult snakes (Tan *et al.*, 1993a; Tan *et al.*, 1993b). Venom production and composition has significant ecological and evolutionary implications, since such a complex mixture of peptides would be very expensive metabolically to produce and therefore would presumably be optimised for maximal biological effect through the processes of natural selection.

Evolutionary Relationship Between Australian Snakes

Of the 142 species of snakes currently identified in Australia, 92 contain both venom glands and fangs, all but one of which (the Brown Tree snake, *Boiga irregularis*) belong to the elapid family (Shea, 1999). Worldwide, the elapid family itself is composed of approximately 61 genera and 300 species and are primarily defined by their unique venom delivery system which entails two permanently erect front- fangs at the end of a relatively immobile maxilla (Keogh *et al.*, 1998). The elapids are further divided into two major lineages on the basis of morphological characteristics: the "palatine erectors" which include the Afro-Asian cobras, Asian kraits and Asian and American coral snakes and the "palatine draggers" such as the terrestrial Australo-Papuan elapids and the hydrophid sea snakes (Smith *et al.*, 1977; McDowell, 1970)

The evolutionary relationship of elapid snakes has previously been based on a range of parameters including internal and external morphology, immunological distances and ecological and biochemical means (Hutchinson, 1990). Recent advances in DNA and protein sequencing technologies have involved the incorporation of molecular means for establishing phylogenetic relationships between snakes, including the inference of species trees from gene trees (Fry and Wuster, 2004; Keogh *et al.*, 1998; Slowinski *et al.*, 1997; Heise *et al.*, 1995). Molecular sequence data may prove to be a powerful tool in that not only can it resolve the order of divergence between species but also give a measure of the timing of that divergence (Wuster *et al.*, 2005). Figure 1.01 details the evolutionary relationship of elapids determined by karyological and electrophoretic data that was later, for the greater part, confirmed by identification of the cytochrome b and 16S rRNA gene sequences (Keogh *et al.*, 1998;

Mengden, 1983). This composite figure also details the clinically relevant Australian elapid genera and whether they are oviparous (egg-laying) or viviparous (producing live young).

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Figure 1.01. Australian elapid snake nomenclature.

(A) Phylogenetic relationship of Australian elapids and **(B)** List of the major *Elapidae* genera with common names, number of species and means of delivering young - oviparous (o) or viviparous (v) (Keogh *et al.*, 1998).

Of the clusters of snake genera detailed in figure 1.01, a number are of particular significance to this study. The brown snake genus *Pseudonaja* is closely related to the *Oxyuranus* or taipan genus. Brown snakes are highly toxic, located over most of Australia and are amongst the most common venomous snakes found in mainland capital cities. Similarly, the inland and coastal taipans (*Oxyuranus microlepidotus* and *Oxyuranus scutellatus* respectively) also posses potently toxic venom, with the inland taipan ranked number one in the list of venomous snakes based on lethal dose, or LD50, scores (0.025mg/kg) upon injection in mice (Broad *et al.*, 1979). Superficially, the two taipan species are similar to brown snakes, occurring in the same areas, but may be differentiated by various scale counts (Shea, 1999). The black snakes or *Pseudechis* species also demonstrate a high degree of relatedness to the taipans and brown snakes. Members of this genus include the mulga or king brown snake, *Pseudechis australis*, and the red-bellied black snake, *Pseudechis porphyriacus*. Also pertinent to this study is the clustering of the viviparous genera *Notechis* (tiger snakes) and

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Tropidechis (rough-scaled snake), which are often confused due to similar banding patterns. Note that the broad-headed genus *Hoplocephalus*, which include the Stephen's banded snake *Hoplocephalus stephensii*, is more distantly related to both of these genera, although bites from all three may be treated with tiger snake antivenom (Sutherland, 1983a).

Clinical Effects of an Elapid Bite

The exact clinical features observed within mammalian prey as a result of a bite from an Australian elapid vary depending on the specific species of snake involved. However, general venom injection results in significant neurological effects including disorientation, flaccid paralysis and respiratory failure in association with multiple coagulopathic effects (Fry, 1999). Untreated bites frequently prove to be fatal, however of the 1,000 to 3,000 bites reported annually in Australia, on average only two result in death (Sutherland and Leonard, 1995). Brown snakes are responsible for the largest number of fatalities in Australian recorded history, with taipans and tiger snakes making up the majority of the remainder (White, 1998). Typically the local effects of the venom at the site of injury are minor, however once in circulation, the systemic effects are of major clinical significance. An exception is the mulga (*P. australis*) whose bite commonly results in local necrosis (Sutherland, 1983b).

Australian elapid snake venoms may be broadly characterised as either procoagulant or non-procoagulant, with significant implications for clinical course and antivenom application (Tan and Ponnudurai, 1990). Examples of snakes with procoagulant venoms are the inland and coastal taipan, common brown snake, tiger snake, Stephen's banded snake, red-bellied black snake and rough-scaled snake. Conversely, elapids with non-procoagulant venoms include the mulga, common death adder and lowland copperhead (Marshall and Herrmann, 1983). The prevailing feature of procoagulant envenomation is massive disseminated intravascular coagulation (DIC) characterised by the disappearance of fibrinogen in the blood via prothrombin activation. This coagulopathy is the leading cause of death in bitten prey. Envenomation from elapids with non-procoagulant venoms may also cause disruptions in haemostasis often as a result of the effects of specific phospholipase A_2 enzymes, or plasmin inhibitors present within the venom (Fry, 1999). Australian elapid venom, most notably from the coastal taipan (*O. scutellatus*), has also been shown to directly effect erythrocyte morphology inducing sphero-echinocytic changes with fragmentation (Arthur *et al.*, 1991).

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Secondary clinical features resulting from elapid bites include haemotoxicity, myotoxicity, nephrotoxicity and neurotoxicity contributing to the nausea, pain and swelling experienced by snake bite victims. The haemotoxic components of venom that result in haemorrhage and sometimes shock are typically attributed to the phospholipase enzymes, discussed in detail further in this chapter (Francis *et al.*, 1993). The myotoxic effects of elapid venoms are relatively insignificant, with the notable exception of the mulga whose envenomation results in severe skeletal muscle damage and necrosis evidenced by pain, tenderness, increased serum creatine kinase levels and myoglobinuria (Harris *et al.*, 2003; Ponraj and Gopalakrishnakone, 1995; Mebs *et al.*, 1983). Again the myotoxic effects of the venom have been attributed, at least in part, to phospholipases. Perhaps the most significant secondary clinical feature of Australian elapid envenomation is neurotoxicity attributed to the blockage of pre- and post-synaptic membranes of nerve and muscle fibres by certain phospholipases and short peptidic neurotoxins. These molecules bind to nicotinic acetylcholine receptors and inhibit their transmission resulting in the pain and flaccid paralysis experienced by victims of an elapid bite (Fry, 1999).

Antivenom is the primary therapy for the majority of medically significant envenomations in Australia and is currently supplied through a single source, the Commonwealth Serum Laboratories (CSL) in Melbourne. All current CSL antivenoms are a $F(ab)^2$ equine liquid product. Up to five hundred cases of snake bite require antivenom each year with the majority of cases reported in rural areas (White, 1998). Treatment with antivenoms have been shown to reverse the neurotoxic effects of Australian elapid venoms, however coagulopathy still remains a primary concern and is the focus for supportive treatment. Envenomation from an Australian elapid may be treated with one of five antivenoms, depending on which species is responsible for the bite, including CSL brown snake, tiger snake, black snake, taipan and death adder antivenoms. Of these, tiger snake antivenom has the greatest range of action, being effective against *Notechis*, *Austrelaps*, *Tropidechis*, *Hoplocephalus*, *Rinocephalus* genera as well as some of the *Pseudechis* species (Crachi *et al.*, 1999; White, 1998).

Characterised Components of Australian Elapid Venoms

As alluded to earlier, snake venoms are a complex mixture of proteins and other constituents, the combined effect of which contributes to the severe homeostatic imbalances observed in

bitten prey. Given the relatively high degree of toxicity of Australian elapid venoms, there has been a significant amount of research into the biochemical and physiological properties of their constituents, and the venom as a whole. Interestingly, there has been comparatively very little study into these toxins at the molecular level. Traditionally, components of Australian elapid venoms have been divided into just three groups: the prothrombin activating factors, the neurotoxins and the phospholipase enzymes, the latter receiving the greatest research attention (Fry, 1999). More recently however, a plethora of other toxins within elapids and other venomous families are becoming apparent as advances in DNA technologies make it easier to screen for these less abundant constituents. Following is a review of the known components of the venom of Australian elapids and other species as relevant to this thesis.

Prothrombin Activating Factors

One of the distinguishing features of Australian elapids compared to all other venomous species of snake is that the procoagulant effects of their venom are limited to prothrombin activation alone: they contain no thrombin-like enzymes (Chester and Crawford, 1982). This is due to the presence of prothrombin activators within the venom that are structurally and functionally similar to mammalian blood coagulation factor Xa.

Haemostasis encompasses all processes resulting in the arrest of bleeding from a ruptured vessel, including reflex vasoconstriction, platelet aggregation, blood coagulation and subsequent dissolution of the clot via fibrinolysis (Davie *et al.*, 1991). One of the key reactions in the mammalian blood coagulation and haemostatic process is the cleavage of the zymogen prothrombin to thrombin which in turn activates fibrinogen to fibrin, eventually resulting in clot formation. The proteolytic cleavage of prothrombin is mediated by the prothrombinase complex, a multimeric complex composed of factor Xa in association with its non-enzymatic cofactor, factor Va, in the presence of calcium on a negatively charged phospholipid bilayer (figure 1.02) (James, 1994; Jackson and Nemerson, 1980). Human activated factor X (Xa) is a two-chained serine protease linked via a single disulphide bond, with a 52 amino-acid glycopolypeptide that is released upon activation. The presence of a vitamin K dependent γ-carboxyglutamic acid residue rich region at the N-terminus of the light chain plays a critical role in the binding of phospholipids and calcium ions, whilst the heavy chain contains the catalytic site residues (Persson *et al.*, 1993; Jackson, 1984).

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Figure 1.02. Schematic representation of the mammalian prothrombinase complex.
Xa = Factor Xa. L = Light chain. H = Heavy chain. Va = Factor Va. PT = Prothrombin.
Circles = calcium ions. Y structures = γ-carboxyglutamic acids (James, 1994).

Prothrombin activators from snake venoms are currently classified on the basis of their cofactor requirement (table 1.01) (Kini *et al.*, 2001a). Metalloproteinases that convert prothrombin to meizothrombin, such as ecarin A isolated from *Echis carinatus*, are classified as either group A (requiring no cofactors for activity) or group B (requiring calcium only for activity) (Morita and Iwanaga, 1978). Group C prothrombin activators are serine proteases that do not require mammalian coagulation factor Va for the cleavage of prothrombin as they contain their own non-catalytic factor Va-like molecule complexed with a factor Xa-like protein. As in the case for the mammalian prothrombinase complex, the factor Va-like subunit of the snake prothrombin activator serves to increase proteolytic activity (Rao and Kini, 2002). Examples of group C prothrombin activators include pseutarin C (isolated from the venom of the common brown snake, *P. textilis*) and oscutarin C (present in the venom of the coastal taipan, *O. scutellatus*) (Masci *et al.*, 1988; Walker *et al.*, 1980). Similarly, group D prothrombin activators are also serine proteases, however they require calcium, phospholipids and mammalian coagulation factor Va for maximal activity, and include the proteases trocarin D (from the rough-scaled snake, *T. carinatus*), hopsarin D (from the Stephen's banded snake, *H. stephensii*) and notecarin D (identified in the venom of the mainland tiger snake, *N. scutatus*) (Weinstein *et al.*, 2001; Marsh *et al.*, 1997; Tans *et al.*, 1985). Interestingly, the prothrombin activators identified from Australian elapid snakes appear to be limited to groups C and D only.

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Table 1.01. Classification of snake venom prothrombin activators. (Kini *et al.*, 2001a).

Studies on the prothrombin activating properties of Australian snake venoms have to date focussed primarily upon purified fractions of the native protein, drawing attention as the only non-mammalian and non-hepatic source of molecules capable of activating prothrombin (Joseph *et al.*, 1999). Recently however, both the factor X-like and much larger factor V-like non-enzymatic unit of pseutarin C, the prothrombin activator present in the venom of *P. textilis*, have been isolated and cloned (Rao *et al.*, 2004; Rao *et al.*, 2003b). Pseutarin C comprises approximately 40% of the total dry weight of the venom of *P. textilis* and its injection results in massive disseminated intravascular coagulation within the body of its prey, ultimately resulting in death (Masci *et al.*, 1988). Its factor X-like component demonstrates similar structure to that of mammalian coagulation factor X with a propeptide region, and light and heavy chains bound by a single disulphide and separated via an activation peptide that is cleaved post-translationally. Overall there is a 48% amino acid identity with human factor X. It has been shown that cleavage of this activation peptide is critical for correct folding of the protease, and that activity is dependent on post-translational glycosylation and γ-carboxylation (Filippovich *et al.*, 2005; Joseph *et al.*, 2003; Baugh and Krishnaswamy, 1996). The non-enzymatic unit of pseutarin C also contains a similar domain structure to its mammalian counterpart with a typical A1-A2-B-A3-C1-C2 domain architecture, in the presence of a number of unique post-translational modifications (Rao *et al.*, 2003b).

Neurotoxins

The nicotinic acetylcholine receptor (nAChR) family are ligand-gated, cation-selective ion channels that span the cell membrane of nerves and muscle fibres. They consist of various pentameric assemblies of structurally related subunits that consist of the agonist binding α

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subunits (α1-α9) and other subunits including β, γ, δ and ϵ (Colquhoun and Patrick, 1997). Skeletal muscle AChRs typically adopt one of two stoichiometries: (α1)$_2\beta\gamma\delta$ or (α1)$_2\beta\epsilon\delta$ (Stroud *et al.*, 1990).

Australian elapid α-neurotoxins (also known as curaremimeteic toxins) are known to bind with high affinity and block postsynaptic skeletal (α1) acetylcholine receptors, typically resulting in inhibition of neuromuscular transmission (Fry, 1999). They are defined as either short or long chain neurotoxins that, although displaying relatively similar function, differ in length and the number of internal disulfide bonds. Mature short chain α-neurotoxins are typically 60-62 amino acids in length with four disulfide bridges, whilst long chain α-neurotoxins have five internal disulfide bonds and are 66-79 (average 73) residues in length (Tsetlin, 1999). The only major difference in primary structure between the two groups is at the C-terminus. Other than variation in the kinetics of association/dissociation with AChRs, the other key functional difference between the two types of α-neurotoxins is that only long chain α-neurotoxins potently block α7 homo-oligomeric neuronal AChRs (Servent *et al.*, 1997).

Snake α-neurotoxins belong to the three-finger toxin family. This non-enzymatic protein family is found only in the venom of elapids and hydrophids (sea snakes) and not in those of vipers and crotalids. They are cysteine rich, a recurring theme in many venom proteins, and display a similar pattern of folding: three β-stranded loops extending from a central hydrophobic core containing the conserved disulfide bridges (figure 1.03) (Golovanov *et al.*, 1993). Although structurally very similar, the three-finger toxin family displays a wide functional diversification, and include not only α-neurotoxins but also κ-bungarotoxins (inhibitors of neuronal nicotinic receptors), muscarinic toxins, fasiculins (inhibit acetylcholinesterase), calcisptins (blockers of L-type calcium channels) and cardiotoxins (form pores in cellular membranes) (Jerusalinsky and Harvey, 1994; de Weille *et al.*, 1991; Grant and Chiappinelli, 1985). α-Neurotoxins also differ from other three finger proteins in that they almost always occur in monomeric form, whereas the other peptides have a tendency towards oligomerisation (Tsetlin, 1999). The functional site of α-neurotoxins has previously been investigated via chemical and site directed mutagenesis. Detailed analysis of the short chain neurotoxin, erabutoxin A, and a long chain neurotoxin, α-cobratoxin (identified in the sea snake *Laticauda semifasciata* and the cobra *Naja kaouthia* respectively) reveal that

neurotoxins recruit a common core of residues for binding and additional residues to determine their specificity of function (Antil *et al.*, 1999; Tremeau *et al.*, 1995).

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Figure 1.03. Spatial structure of a three-finger neurotoxin.

The spatial structure was derived from neurotoxin II from the central Asian cobra *Naja naja oxiana*. Note the heavily disulfide bonded central core at the top of the protein and three "finger" loops formed by descending β-sheets (Golovanov *et al.*, 1993).

Short chain α-neurotoxins identified in the venom of Australian elapids are highly homologous, basically charged post-synaptic blockers of neuromuscular transmission. They have a typical disulfide bonding pattern of 1-3, 2-4, 5-6, 7-8 and contribute to the peripheral muscle paralysis observed in bitten prey. An example of short α-neurotoxins identified in an Australian elapid include taipan toxins 1 and 2 isolated from the venom of the coastal taipan *O. scutellatus* (Zamudio *et al.*, 1996). The taipan toxins were observed to inhibit the binding of α-bungarotoxin to nicotinic AChRs (although at a reduced affinity) but not to central neuronal receptors. They also demonstrate a remarkably high degree of conservation to another short chain neurotoxin isolated from the mulga *P. australis*, termed Pa-a, as well as other short chain neurotoxins from the venom glands of a number of sea snakes (Takasaki and Tamiya, 1985). Probably the most well characterised short neurotoxins from an Australian terrestrial elapid are a family of toxins from the common brown snake, whose genomic sequences have been cloned and the recombinant proteins expressed for functional characterisation (Gong *et al.*, 2000; Gong *et al.*, 1999). They contain a 21 amino acid, hydrophobic signal peptide and 6 different isoforms of mature protein 57-58 residues in

length. The genomic sequence has also been shown to undergo an accelerated rate of evolution, a feature common to toxin genes and dealt with in more detail later in this chapter.

Long chain α-neurotoxins also have a highly conserved disulfide bonding pattern of 1-3, 2-4, 5-6, 7-8, 9-10 (note the additional bond, present within the central loop II), but are not all basic and have a greater variance in sequence (Fry, 1999). The stability of the additional bond in long chain toxins has been linked with their ability to bind α7 AChRs (Servent *et al.*, 1997). Again there are a number of examples of previously identified long chain α-neurotoxins from the venom of Australian elapids, including peptides isolated from the death adder, *Acanthophis antarctius*, tiger snake, *N. scutatus*, mulga, *P. australis* and common brown snake, *P. textilis* (Sheumack *et al.*, 1990; Takasaki, 1989; Tyler *et al.*, 1987; Halpert and Eaker, 1975). The later, pseudonajatoxin b from the common brown snake, has been demonstrated to be a highly lethal blocker of nicotinic acetylcholine receptors with a 21 amino acid signal peptide and a mature protein structure homologous to the traditional long chain neurotoxin moiety (Gong *et al.*, 2001). Recent evidence suggested by Fry *et al.* (2003) indicates that the nomenclature of α-neurotoxins should be adjusted to group I (short chain neurotoxins), group II (long chain neurotoxins) and group III (neurotoxins similar to those as described from the *Pseudonaja* genus by Gong *et al.* (1999)) to encapsulate the subtle variations in primary structure demonstrated by this toxin family.

Phospholipase A$_2$ Enzymes

Phospholipase A$_2$ (PLA$_2$) enzymes specifically hydrolyse the *sn*-2 ester bond within the backbone of glycerophospholipids releasing lysophospholipids and free fatty acids, typically in a calcium dependent manner (van den Bosch *et al.*, 1965). They have a ubiquitous role in mammalian systems where their actions are generally non-toxic, including cell proliferation, fertilisation, membrane homeostasis and smooth muscle contraction (Kini, 2003). PLA$_2$s are the most widely studied of all phospholipases, which are divided into four groups, A$_1$, A$_2$, C and D, on the basis of their specificity of cleavage.

Phospholipase A$_2$ enzymes present within venom display similar primary structure and catalytic function with their mammalian counterparts, however, in contrast to mammalian enzymes, venom PLA$_2$s may be highly toxic with a plethora of pharmacological effects.

Phospholipases are found in almost all snake venoms, regardless of genera, and it is the PLA_2 enzymes of the Australian elapids that constitute the single most widely studied family of toxins from these snakes (Harris, 1997). Venom PLA_2s are medium sized (119-143 amino acids), heavily disulfide bonded proteins that are classified into three groups on the basis of primary structure and disulfide connectivity: group I, isolated from elapid and hydrophid venoms, group II, present within vipers and crotalids and group III, which are found in lizard and bee venoms (Arni and Ward, 1996). Australian elapid group I PLA_2s are typically 13kDa, basic proteins with seven disulfide bridges in a conserved pattern of 1-8, 2-14, 3-5, 4-13, 6-12, 7-10 and 9-11. The non-toxic phospholipase activity and toxic enzymatic activity are suspected to occur in different active sites of the molecule (Fry, 1999).

Besides being implicated in the digestion of prey, venom PLA_2s have been associated with a wide range of physiological effects including haemorrhagic, myotoxic, haemolytic, hypotensive, oedema forming, platelet aggregating, convulsant, cardiotoxic and pre- and post-synaptic activities (Arni and Ward, 1996). This variation in activity is despite what appears to be a high degree of conservation in their primary structure, and may be independent of phospholipase catalytic activity. Typically the venom from a single snake contains multiple PLA_2 isoforms. Fry (1999) clusters the group I Australian elapid PLA_2s on the basis of their pharmacological activities into five types: haemotoxic, myotoxic, neurotoxic and non-toxic (either enzymatically active or inactive).

The haemotoxic PLA_2s have haemorrhagic, anti-platelet aggregating and anticoagulant effects in mammalian prey by specifically binding to components within the coagulation cascade resulting in disruptions of haemostasis. Examples include phospholipases isolated from the venoms of the lowland copperhead (*Austrelaps superbus*) and tiger snake (*N. scutatus*) (Francis *et al.*, 1995; Yuan *et al.*, 1993). Although Australian elapids are not noted for their myotoxic venoms, there are two exceptions in the mulga (*P. australis*) and Collet's snake (*Pseudechis colletti*), which may induce severe muscle damage and necrosis with associated myoglobinuria and myoglobinuric nephropathy (Dambisya *et al.*, 1995; Weinstein *et al.*, 1992; Mebs *et al.*, 1983). The myotoxic properties of the mulga have been attributed, at least in part, to a number of isoforms of basic PLA_2s that damage muscle fibres by blocking K^+ conductance resulting in loss of contractility (Fatehi *et al.*, 1994; Geh *et al.*, 1992).

The Australian elapid neurotoxic phospholipases may occur as either single chain molecules or associated in multimeric complexes. They are typically basic and have a presynaptic mode of activity. Of the single chain neurotoxic phospholipases, notexin from the tiger snake (*N. scutatus*) is the most thoroughly characterised (Chang *et al.*, 2004). Although it has weak phospholipase activity, notexin acts to block neuromuscular transmission by reducing the available amount of intracellular acetylcholine at the motor nerve terminal via the mobilisation of vesicular acetylcholine (Mollier *et al.*, 1990; Harris *et al.*, 1973). Notexin has also demonstrated a direct myotoxic effect. Single chain presynaptic neurotoxins with PLA_2 homology have also been identified in other snake venoms including pseudexins from the red-bellied black snake (*P. porphyriacus*), scutoxins also from the tiger snake and OS_1 and OS_2 from the coastal taipan (*O. scutellatus*) (Lambeau *et al.*, 1995; Francis *et al.*, 1991; Schmidt and Middlebrook, 1989).

A common trend in Australian elapids is for neurotoxic PLA_2s to occur in multimeric complexes either with additional phospholipases or with other peptidic components such as serine protease inhibitors or α-neurotoxins (Fry, 1999). Examples of both are evident in the venom of the coastal taipan (*O. scutellatus*). Taipoxin is a potent blocker of acetylcholine release from the presynaptic membrane of the nerve terminal. It is composed of three non-covalently bound PLA_2 chains, α, β and γ, all with variable activity in a 1:1:1 stoichiometry. Only the α-chain displays neurotoxic activity, whilst the β-chain has surprisingly demonstrated mitogenic properties (Lipps, 2000; Lind and Eaker, 1982; Fohlman *et al.*, 1976). A homolog of taipoxin, paradoxin has been identified in the most toxic snake venom in the world, that of the inland taipan (*O. microlepidotus*) (Fohlman, 1979). A multimeric toxin (5 subunits) composed entirely of PLA_2 enzymes (textilotoxin) has also been identified from the venom of the common brown snake (*P. textilis*). Textilotoxin is a potent presynaptic myoneurotoxin that has the greatest lethality in mice of any identified snake neurotoxin (Pearson *et al.*, 1993; Su *et al.*, 1983). Finally, the venom of the coastal taipan also contains an example of a multi-chain neurotoxic complex comprised of a PLA_2 enzyme in the presence of non-homologous peptides called taicatoxin. Taicatoxin is a specific blocker of high threshold calcium channels in the heart, as well as a potassium channel blocker in the brain (Doorty *et al.*, 1997; Brown *et al.*, 1987). It contains an alpha-neurotoxin, serine protease inhibitor as well as a PLA_2 enzyme, all with varying activities (Possani *et al.*, 1992b).

Australian elapid venoms also contain a number of PLA_2 enzymes that display no direct toxic activity, irrespective of the presence or absence of phospholipase activity, for example the β-chain of taipoxin. Although the role of these proteins within the venom has yet to be elucidated (evidence suggests they may act as a chaperone to assist binding of the toxic component to its specific molecular target, or aid in the process of digestion), they may help in the identification of functionally important residues within the sequences of active PLA_2s (Kini, 2003). Despite being the most widely studied component of Australian snake venoms, much work still remains in the study of phospholipases, including the determination of functional sites within active molecules, cataloguing the full physiological activity of each molecule, plus a comprehensive study on the number of isoforms of PLA_2s in individual elapid species.

Venom Natriuretic Peptides

The natriuretic peptide family, found to occur naturally in mammalian systems, play a significant role in a number of physiological processes including cardiovascular, renal and endocrine homeostasis. A structurally related but genetically distinct family, the natriuretic peptides are characterised by a homologous 17 amino acid disulfide ring (figure 1.04) (Chen and Burnett, 2000). Despite differences in primary structure, mammalian natriuretic peptides differ in their tissue of origin and specific molecular targets. Both atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are produced in myocardial cells (although BNP was originally isolated from porcine brain, hence the nomenclature), and both bind the natriuretic peptide-A receptor (NPR-A). Binding to the receptor on the target organ stimulates the production of the second messenger cyclic GMP with the flow on effects including natriuresis (increased urine volume), vasodilation, antimitogenesis and renin inhibition (Misono, 2002). C-type natriuretic peptide (CNP), which lacks the C-terminal extension, is expressed predominantly within the central nervous system and vascular endothelial cells. On binding to natriuretic peptide-B receptors (NPR-B), its effects are strongly vasoactive, with little effect on natriuresis (Koller *et al.*, 1991). A third type of receptor (NPR-C) has also been identified and shown to bind all three natriuretic peptide subtypes, and has been implicated in the control of the local concentration of natriuretic peptides to the other receptors (Maack, 1992).

Figure 1.04. Natriuretic peptide structures.

Amino acid sequence and structure of human atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP) and dendroaspis natriuretic peptide (DNP) (Richards *et al.*, 2002).

Natriuretic peptides have also been isolated from the venom of a small number of snakes including both elapids and vipers (Fry *et al.*, 2005; Amininasab *et al.*, 2004). The most widely studied of these is a peptide from the venom of the green mamba (*Dendroaspis angusticeps*) called DNP (figure 1.04) (Schweitz *et al.*, 1992). This 38 amino acid peptide has been shown to have structural and functional similarities to its mammalian counterparts, particularly ANP and BNP. DNP is a potent simulator of natriuresis, diuresis and vasorelaxation (Best *et al.*, 2002; Lisy *et al.*, 1999). Despite keen research interest into this molecule, the full-length DNP gene has not been cloned from the green mamba or any other related snake (Pan *et al.*, 2004). Recently, Fry *et al*. (2005) demonstrated the presence of a number of isoforms of novel natriuretic peptides, 35-39 amino acids in length, present in the venom of taipans and called TNPs. This is the first report of the presence of a natriuretic peptide in the venom of an Australian elapid. The multiple functions displayed by natriuretic peptides make them ideal candidates for pharmacological therapy of cardiovascular disease.

Pseudechetoxin and Pseudecin

Pseudechetoxin, and its related homolog pseudecin, are peptidic toxins that target cyclic nucleotide-gated (CNG) ion channels and have been isolated from the venom of the mulga (*P. australis*) and red-bellied black snake (*P. porphyriacus*) respectively (Brown *et al.*, 1999). CNG ion channels play a central role in signal transduction in retinal photoreceptors and olfactory neurons, and have been identified in a number of other tissues in the body including the brain, heart and kidneys. They modulate the membrane potential of the cell and intracellular calcium levels in response to stimulus-induced changes in cyclic nucleotide concentration (Burns and Baylor, 2001; Bradley *et al.*, 1997). Pseudechetoxin binds to the pore turret of CNG ion channels and inhibits the flow of current, the first peptide toxin known to have this function (Brown *et al.*, 2003). Both the full-length cDNA sequence of pseudechetoxin and pseudecin have been cloned and have been shown to bind to CNG channels with different affinities despite a high degree of homology in primary structure (Yamazaki *et al.*, 2002). Pseudechetoxin is heavily disulfide bonded and related to the cysteine-rich secretory protein (CRISP) family as well as the toxin helothermine, a calcium channel blocker isolated from the venom of the Mexican bearded lizard (*Heloderma horridum*) (Nobile *et al.*, 1996). Pseudechetoxin is a prime example of a novel toxin that has been used to study the pharmacology of a mammalian system/target, and Australian elapid venoms may serve as a rich source of other such proteins.

Other Venom and Venom Gland Proteins

Although the primary study of Australian elapid toxins has focussed in just three major areas, the prothrombin activating factors, the neurotoxins and the phospholipases, more and more evidence of other biologically significant molecules within the venom of these snakes is coming to light. Examples other than the natriuretic peptides and pseudechetoxin of unique elapid venom components include *textilinin* isolated from the common brown snake (*P. textilis*) and related proteins from other Australian snakes (Masci *et al.*, 2000). Textilinin is a plasmin inhibitor with antihaemorrhagic properties and is capable of stemming blood loss in a murine tail vein bleeding model (Filippovich *et al.*, 2002). Its exact role as a venom toxin still remains unclear.

A protein with *L-amino acid oxidase* (LAAO) activity has also been isolated from the venom of the mulga (*P. australis*) and demonstrated to have antibacterial effects (Stiles *et al.*, 1991). This family of proteins convert L-amino acids into keto acids, ammonia and hydrogen peroxide, the later product proving to be a potent bactericide. It is speculated that LAAOs present within the venom help aid against putrification of the prey, which may be contaminated with pathogens such as *Aeromonas*, during the long period of digestion within the snake (Thomas and Pough, 1979). Although the amino acid composition or gene sequence for the mulga LAAO still remains undetermined, homologs in other snakes including a number of vipers have previously been cloned and shown to have antibacterial and platelet aggregating effects (Stabeli *et al.*, 2004; Takatsuka *et al.*, 2001).

An array of other toxin and protein sequences have also been identified from the venom of *Viperidae* and other non-Australian elapid snake species, which may very well prove to be present in the venom of Australian snakes. Examples include the *waprins*, which currently have a poorly defined function within the venom, *vascular endothelial growth factors* such as those identified in the venom of a number of vipers and shown to increase vascular permeability and *cysteine proteinase inhibitors* that may protect venom components from host proteolytic cleavage (Torres *et al.*, 2003; Mashiko and Takahashi, 2002; Junqueira de Azevedo *et al.*, 2001). Other proteins identified in the venom of snakes other than Australian elapids also include *C-type lectins*, which have been postulated to have anticoagulant and even anti-tumorigenic effects, disintegrins with a wide range of pharmaceutical applications, as well as proteins with structural homology to *nerve growth factors* (McLane *et al.*, 2004; Morita, 2004; Kashima *et al.*, 2002).

Interestingly, the study of snake venoms is not just limited to the components within the venom themselves, but also entails research into proteins within the venom gland responsible for the manufacturing and transport of toxins. An excellent example is calglandulin; a putative calcium binding protein identified from the venom gland of the Island jararaca, *Bothrops insularis*, which displays structural homology to the calmodulin family of proteins. Calglandulin has been implicated in the export of toxins out of the venom gland cells and into the venom itself (Junqueira-de-Azevedo Ide *et al.*, 2003). So in summary, the venoms of Australian elapids snakes are a rich source of protein and polypeptide molecules that exert pharmacological effects in mammalian systems, the details of many of which have yet to be determined.

<u>Evolution of Toxins</u>

There are approximately 3,200 species of snake identified worldwide, 1,300 of which produce toxic venoms (Phui Yee *et al.*, 2004). Animals may acquire toxicity either by metabolic synthesis of secondary metabolites taken up from external sources or via *de novo* expression of proteins from specific toxin genes (Mebs, 2001). The diversity of proteins observed in snake venom is a direct result of their mode of evolution. Toxin genes are subject to frequent duplication events within the snake genome, often followed by functional and structural diversification at an accelerated rate, as evidenced by relatively conserved intronic sequences compared to gene exons (Kordis and Gubensek, 2000; Moura-da-Silva *et al.*, 1995). Evidence of multiple gene duplication events to form a large multigene family are evident when examining the phylogeny of the three-finger toxin family (Fry *et al.*, 2003). It has been postulated that these rates of accelerated evolution have been driven by the necessity for the snake to kill and digest its quarry, or to overcome the development of further resistance within prey (Kini and Chan, 1999).

An understanding of the evolution of snake multigene families has important implications for the predication of the occurrence and activity of similar toxins in related taxonomic groups whose venom has not been characterised on such a detailed level. Similarly, cataloguing toxin gene trees will serve to highlight evolutionarily distinct or novel proteins that may serve in unique research, diagnostic or pharmacological applications (Fry *et al.*, 2003). A systematic study of a venom group I PLA_2 genomic sequence from the spitting cobra (*Naja sputarix*) has already confirmed the theory of adaptive evolution of toxic venom components (Jeyaseelan *et al.*, 2000). These peptides serve as a useful tool in the study of toxin evolution as they are so widely distributed throughout venomous snakes worldwide.

Snake venoms display a wide diversity of toxins that vary both in structure and function, as evidenced by this review. These enzymatic and non-enzymatic proteins target a large number of mammalian systems, resulting in multiple organ failure and often death in bitten prey. Multiple isoforms of the one protein also may exist in a single venom, for example the phospholipases and neurotoxins, and it has been suggested that these variants allow the snake to adapt rapidly to different prey or changes in environment. These isoforms may arise from point mutations, gene duplications and recombinations and alternative post-translational

modifications of a single toxin gene, which are then subject, in part, to natural selection (Mebs, 2001).

Interestingly, elapids have evolved novel mutations within the nicotinic acetylcholine receptor of their own nervous system that selectively recognises acetylcholine but is insensitive to the lethal neurotoxins secreted in their own venom (Ohana *et al.*, 1991; Neumann *et al.*, 1989). Conversely, inhibitors in the snake's sera provide protection against phospholipase A_2 enzymes that may be potently neurotoxic or myotoxic (Hains *et al.*, 2001; Thwin and Gopalakrishnakone, 1998). In this manner Australian elapids have coevolved the ability to protect themselves against the components that have been recruited into their venom.

Venoms as Therapeutics

The study of snake venom toxins has previously focussed on a number of different objectives, including the discovery of individual components in the venom and determination of their mechanisms of action, the identification of a means to neutralise the adverse toxic effects of the venom in a bitten subject, the production of research and diagnostic tools based on venom constituents to study physiological processes and finally the development of therapeutic and pharmaceutical agents based on the structure of toxins (Kini, 2002). Venoms are a unique source of molecules that may be of therapeutic potential as they arise from a non-mammalian source, yet often specifically target functional mammalian systems in a well characterised manner. They are similarly useful in the drug discovery process due to their high potency and selectivity and have the advantage that often they are relatively small peptides that may act as a three-dimensional scaffolds to deliver a specific pharmacological effect (Harvey *et al.*, 1998). Venom peptides also have an inherent degree of stability, an advantage for a therapeutic agent, due to their necessity to survive chemical and enzymatic degradation from host tissues, as well as other components within the venom itself. This stability often arises from the heavily disulfide bonded structure of many toxins, or from unique post-translational modifications (Lewis and Garcia, 2003).

Factors isolated from snake venoms have been used for therapeutic purposes for a number of decades. The first clinically controlled experiments using snake venom were performed in 1934, when the crude venom of a Russell's viper was used as a clotting agent for the treatment of haemophilia (MacFarlane and Barnett, 1934). More recently, thrombin-like

enzymes have been used as anticoagulants for the treatment of venous thrombosis, a condition responsible for significant morbidity including venous thromboembolism. An example of such a thrombin-like enzyme is batroxobin from *Bothrops atrox* venom (Stocker and Barlow, 1976). Examples of the putative application of an Australian snake venom toxin as a therapeutic lie in the textilinins, which are being studied as an anti-bleeding agent (Filippovich *et al.*, 2002). Given the wide array of physiological effects in the mammalian system, Australian elapid toxins may yet prove to be a unique and diverse source of molecules that could be applied in a diagnostic or therapeutic setting.

Project Aims and Significance

Despite demonstration of the relatively high degree of toxicity of Australian snake venoms, they have remained largely understudied at the molecular level, with relatively little known about the clinical pathology of the whole venom or various activities of individual components (Fry, 1999). This is evidenced by the fact that although there are over nine million nucleotide entries and 250,000 protein entries in the National Centre for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/) for humans alone, there are only 7gene entries and 20 peptide submissions for the inland taipan (*O. microlepidotus*), current as of February 2005. This is remarkable given that this elapid contains the most potently toxic venom of any snake in the world, although in part it may be attributed to the difficulty in obtaining venom glands to perform molecular studies. Similarly low numbers are also observed in the other Australian elapids involved in this study.

*So, given the diversity of proteins and polypeptides known to exist within the venoms of Australian snakes, and given the relative potency of these components in altering mammalian functional systems and as yet their relatively unstudied nature, this study was aimed at identifying and further characterising those components at a molecular level within the venom of the coastal taipan (*O. scutellatus*) and related Australian elapids which may be of diagnostic or therapeutic benefit*. The primary aims of this study were two-fold; firstly to further characterise the prothrombin activator from the coastal taipan and related species including cloning of the mRNA transcript of this protein and expression of recombinant form for functional studies. A secondary aim was the establishment a cDNA microarray from the venom gland mRNA of the coastal taipan for the purposes of cross species comparisons of

gene expression levels and the identification of novel compounds which may be of pharmacological interest.

Blood loss associated with significant trauma is a major cause of death. A group of agents known as surgical tissue adhesives have been developed to promote haemostasis and tissue sealing during surgery. "Fibrin glues" are the most effective tissue adhesives currently available (Jackson, 2001). They are composed of two separate solutions of fibrinogen and thrombin which, when mixed together, result in a cross-linked fibrin patch to stem bleeding at the site of injury. However, the components of the applied topical mix must be purified from human or animal sources, raising the potential for the transfer of infectious agents to the patient, such as hepatitis B and C viruses and HIV (Thompson *et al.*, 1988). Recently, human prothrombin has been cloned and expressed, and may be efficiently converted to α-thrombin by the prothrombin activator isolated from *O. scutellatus*. Cloning and expression of the components of this prothrombin activator will facilitate production of large quantities of recombinant α-thrombin for therapeutic use and will simultaneously eliminate the potential susceptibility to pathogens from animal and human sources. *Hence, this project will focus on the characterisation, cloning, expression and eventual trialing of the factor Xa-like serine protease from the prothrombin activator of an Australian elapid*. The advantage of such a protease is its rapid mechanism of action, its stability under *in vivo* conditions, the small dose required to elicit a large response, and its ability to convert thrombin from prothrombin in the absence of any other cofactor required by mammalian prothrombinase.

Similarly, analysis of the microarray and corresponding cDNA library will provide molecular evidence for other components within the venom of Australian snakes that can then be further investigated as pharmacological tools. Results will provide a systematic comparison of those components within the venom gland of these snakes and may also shed light on the evolutionary relationship between species. To this end, venom glands were collected from a total of 8 Australian elapids representing a range of genera. A comparative analysis of the microarray data should not only aid in the identification of novel compounds that may be of therapeutic benefit but also provide information for the detection and treatment of snake bite.

CHAPTER 2

Comparative Analysis of Prothrombin Activators from the Venom of Australian Elapids

Snake venoms contain a large number of proteins known to affect the mammalian haemostatic mechanism via coagulant, anticoagulant and/or fibrinolytic actions. The coagulant effects of snake venom were first recorded in 1781, when it was noted that injection of viper venom into the jugular vein of rabbits resulted in rapid blood coagulation, followed immediately by death (Fontana, 1781). Since then, well over a hundred different snake venoms have been identified as affecting the haemostatic system through a variety of mechanisms (Markland, 1998).

Snake venom constituents may affect the haemostatic mechanism in either an enzymatic fashion (including serine proteases, metalloproteinases, phospholipases and nucleotidases) or in a non-enzymatic manner (including disintegrins and C-type lectins) (Braud *et al.*, 2000). In conjunction with their ability to interrupt the haemostatic system, snake venom proteins may also serve to increase target tissue permeability to other venom toxins which can then interfere with the processes of neurotransmission as a method of disabling their victims (Faiz *et al.*, 1996). Although haemostatically active snake venom proteins may have a specific coagulant or anticoagulant mechanism of action, often this distinction is not clearly defined, with some proteins displaying both activities depending on domain structure, venom concentration and time-course of envenomation, whilst others have opposing actions *in vivo* as compared to *in vitro* studies (Markland, 1998; Reid and Chan, 1968). This has led to some degree of confusion within the literature regarding classification, however haemostatically active venoms may be broadly grouped into four classes: 1) venoms affecting the blood coagulation cascade, 2) venoms affecting platelet aggregation, 3) venoms affecting plasmin mediated fibrinolysis and 4) haemorrhagic venoms (Marsh, 1994).

The predominant haemostatic activity described in the venom of Australian elapid snakes falls into the first of these groups: a direct affect on the blood coagulation cascade. Interestingly, the procoagulant effects of Australian elapid venoms are limited to prothrombin activation alone, with no direct activation of fibrinogen (Chester and Crawford, 1982). This is due to the presence of prothrombin activators that are structurally and functionally similar to mammalian coagulation factor X. As previously described in chapter 1, snake venom prothrombin activators are divided into four categories on the basis of their cofactor

requirement for activity with those found in Australian snakes limited to groups C and D (Kini *et al.*, 2001a).

The initial aim of this study was to further characterise the prothrombin activator complex at the cDNA level from a representative group of Australian elapids. It was hoped that a comparative analysis of both the factor X-like component in group C and D snakes and the factor V-like component found only in group C elapids would provide information not only on the evolutionary relationship between Australian elapids, but also further insight into the current classification system of snake prothrombin activators. As the amino acid sequence has not previously been determined for many of these toxins, this study also provides an indication of conserved regions within the protein that may be of structural or functional importance. This investigation was supported by direct examination of the prothrombin activator components within the venom itself. Furthermore, besides contributing to the knowledge base of haemostatically active snake venom proteins, recombinant expression and characterisation of one of these proteases will also aid in the development of a factor Xa-like therapeutic agent. Currently there is an ever burgeoning need for new therapeutic and diagnostic tools, and given their stability, general abundance within venom, wide ranging activities and specificity for molecular targets within mammalian systems, snake toxins are becoming a highly favoured potential source for such molecules.

Methods and Materials

RNA Isolation and First Strand cDNA Synthesis

Venom glands were excised from Australian elapids including the coastal taipan (*Oxyuranus scutellatus*), inland taipan (*Oxyuranus microlepidotus*), common brown snake (*Pseudonaja textilis*), red-bellied black snake (*Pseudechis porphyriacus*), mulga (*Pseudechis australis*), mainland tiger snake (*Notechis scutatus*), rough-scaled snake (*Tropidechis carinatus*) and Stephen's banded snake (*Hoplocephalus stephensii*) collected under Queensland Parks and Wildlife's permit number WISP03122905. Snap frozen glands were partially thawed and homogenised with a polytron and RNA isolated using the Tri Reagent method (Sigma-Aldrich, St Louis, Missouri).

Briefly, homogenised venom gland in Tri Reagent was added to 200μL of chloroform per 1mL of original sample, incubated at room temperature for 15min and centrifuged at 16,100g for 15min at 4°C. The aqueous phase was removed and RNA precipitated with 500μL of isopropanol per 1mL of Tri reagent at room temp for 10min, followed by centrifugation at 16,100g for 10min at 4°C. The RNA was washed with 70% ethanol, re-pelleted via centrifugation, dried briefly at room temperature and resuspended in diethylpyrocarbonate-treated (DEPC) water. Samples were quantitated by measuring absorbance at 260nm, stored at -70°C and used for first strand cDNA synthesis.

First strand cDNA was synthesised from 1μg of total RNA in DEPC-water with 500ng of oligo(dT)$_{12-18}$ primer in a total volume of 12μL (Roche Diagnostics, Basel, Switzerland). The reaction mixture was heated to 70°C for 10min for oligo(dT) annealing and then chilled briefly on ice before incubation with 200 units of Superscript II RNase H$^-$ Reverse Transcriptase (Invitrogen, Mt Waverly, Australia) buffered in a final concentration of 50mM Tris-HCl pH 8.3, 72.5mM KCl, 3mM MgCl$_2$ and 500nM dNTPs (Promega, Madison, Wisconsin). The final reaction volume of 20μL was incubated at 42°C for 60min, then stopped by heating to 70°C for 15min. cDNA samples were finally ethanol precipitated, resuspended in sterile water and stored at -20°C.

Ethanol Precipitation

The general protocol used for all ethanol precipitations was as follows. A total of 2.5 volumes of 100% ethanol was added to either DNA or RNA in water along with 1μL of 3M sodium acetate pH 5.4. The sample was chilled on dry ice for at least 30min and then centrifuged at 16,100g for 30min at 4°C. Supernatant was removed and the pellet was washed with 500μL of 70% ethanol, then respun at 16,100g for 20min at 4°C. The pellet was subsequently air dried at room temperature and resuspended in the appropriate volume of water before storage at either -20°C (DNA) or -70°C (RNA).

PCR of Factor Xa-like Prothrombin Activator

PCR amplification of the full length coding sequence of the Factor X-like protease was attempted on all cDNAs isolated from Australian elapids. An approximately 1.4kb product

was amplified with forward (5'-ATG GCT CCT CAA CTA CTC CTC TG-3') and reverse (5'-TTA GAG CCG ACC AGT GCT TGA CTC-3') primers designed from the recently published *P. textilis* sequence (Genbank accession number AY631238) (Sigma-Aldrich, St Louis, Missouri). The PCR reaction mix was made to a final volume of 25µL containing the following: approximately 200ng of cDNA template; 1 unit of AmpliTaq gold (Applied Biosystems, Foster City, California); 10mM Tris-HCl pH 8.3; 50mM KCl; 2mM $MgCl_2$; 200µM dNTPs (Promega, Madison, Wisconsin) and 50ρmol of each of the forward and reverse primer. All reactions were run with appropriate no template controls.

The reaction mixture was then thermocycled on a GeneAmp^{TM} 2700 PCR machine (Perkin-Elmer, Norwalk, Connecticut) with an initial denaturation at 95°C for 8min, followed by 30 cycles of 95°C for 30sec, 52°C for 45sec and 72°C for 2min, with a final extension of 72°C for 7min. The resulting PCR product was analysed on a 1% Tris-Acetate EDTA (TAE) agarose gel and visualised with ethidium bromide (at a final concentration of 100ng/mL) under UV light. Size comparisons were made with 0.5µg of 1kb DNA ladder (Invitrogen, Mt Waverly, Australia).

Gel Purification and Cloning into pGEM®-T Vector System

PCR bands of interest were excised from the agarose gel and purified with a QIAEX II Gel Extraction kit according to manufacturer's instructions (Qiagen, Hilden, Germany). Briefly, the agarose band was resuspended in 3 times the volume of QX1 buffer in the presence of 10µL QIAEX II beads and 10µL of 3M sodium acetate pH 5.4 at 50°C for 10min. The beads were then pelleted via centrifugation at 16,100g for 30sec and washed once with 500µL QX1 buffer and twice with 500µL Buffer PE. They were then air-dried and the purified PCR product eluted with 20µL of EB buffer at 50°C for 10min followed by recentrifugation to remove the beads. An aliquot of the purified product was rerun on a 1% TAE agarose gel to confirm recovery.

Prior to identification by sequencing, purified PCR products of interest were cloned via the pGEM-T vector system, which utilises the 3'-T overhang incorporated into all PCR products amplified with AmpliTaq gold DNA polymerase (Promega, Madison, Wisconsin). A total of 8µL of purified PCR product was incubated with 50ng of pGEM-T vector and 3 units of T4

DNA Ligase in the presence of 30mM Tris-HCl pH 7.8, 10mM $MgCl_2$, 10mM DTT and 1mM ATP 5% PEG in a final volume of 20μL at 16°C overnight. The ligation mixture was ethanol precipitated as previously described, eluted in 16μL of sterile water and stored at -20°C.

Preparation and Transformation of Competent dH5α Cells

Electrocompetent dH5α *E. coli* cells were prepared by inoculating 4mL of Luria-Bertani (LB) broth (10g trypticase peptone, 5g yeast and 10g NaCl autoclaved in 1L water) with a single colony of dH5α stock, growing at 37°C shaking overnight. This starter culture was then used to inoculate a further 500mL of LB broth, and the cells shaken at 37°C until they reached early to mid-log phase of growth (typically an optical density of 0.5 to 0.8 when reading absorbance at 600nm). The cells were chilled on ice for 15min and harvested by discarding supernatant after centrifugation at 3,300g for 15min. They were then washed gently in 500mL of ice-cold sterile water followed by recentrifugation and a second wash in 250mL of chilled sterile water. A further wash in 10ml of ice-cold 10% glycerol was performed, whereupon the dH5α cells were resuspended in 1.5mL of 10% glycerol, frozen in 80μL aliquots on dry ice and stored at -70°C until use.

A total of 4μL of purified ligation mix was incubated with 40μL of thawed electrocompetent dH5α *E. coli* cells on ice for 1min and transferred to a 0.2cm electroporation cuvette (Bio-Rad, Hercules, California). The Gene Pulser apparatus was then set to 25μF, 200Ω and 2.5kV and the mixture pulsed briefly. The cells were immediately recovered in 1mL of LB broth at 37°C for 60min. Two dilutions of this mixture, typically 50μL and 200μL, were then plated on LB agar plates (1L of LB broth plus 15g agar) and grown at 37°C overnight. In the case of transformations with pGEM-T and other vectors that contained antibiotic resistance and colour selection, the LB agar plates were prepared with 100μg/mL ampicillin, 20mg/ml IPTG and 100mM 5-bromo-4-chloro-3-indolyl-bD-galactoside (X-Gal) resuspended in dimethyl sulphoxide (DMSO). Plates were then examined the following day for white colonies.

Isolation of Plasmid DNA

Positive colonies containing vector inserts were picked and grown in 4mL on LB broth plus 100mg/mL ampicillin at 37°C overnight. Plasmid was then purified using a QIAprep Spin

Miniprep Kit according to manufacturer's instructions (Qiagen, Hilden, Germany). Briefly, 2mL of culture was pelleted via centrifugation at 16,100g for 10min at 4°C and the supernatant decanted. The bacterial pellet was resuspended in 250μL of buffer P1 followed by the addition of 250μL of buffer P2 and 350μL of buffer N3 with gentle inversion in between. The solution was centrifuged for 10min at 16,100g at room temperature and the supernatant applied to a QIAprep column. The column was spun for 30sec and the flow through discarded, followed by washes with 500μL of buffer PB and 750μL of buffer PE. Plasmid DNA was then eluted with 50μL of elution buffer (10mM Tris-HCl pH 8.5) and quantitated by spectrophotomeric readings of absorbance at 260nm.

Plasmids were then examined for the presence of inserts via restriction digest. In the case of pGEM-T vectors, 500ng of purified plasmids were double digested with 10units of both Nco1 and Pst 1 restriction enzymes buffered in 50mM Tris-HCl pH 7.9, 100mM NaCl, 10mM $MgCl_2$, 1mM dithiothreitol plus 100ng of Bovine Serum Albumin (BSA) at 37°C for 60min (New England Biolabs, Beverly, Massachusetts). Digested plasmid was visualised on a 1% TAE agarose gel stained with ethidium bromide to examine if an insert of expected molecular weight was produced.

Sequencing

Sequencing of clones was performed using an ABI Big Dye version 3.1 Terminator cycle sequence ready reaction kit (Perkin-Elmer, Norwalk, Connecticut). Approximately 300ng of plasmid template was added to 2ρmol of either T7 forward primer (5'-TAA TAC GAC TCA CTA TAG GG-3') or M13 reverse primer (5'-GGA AAC AGC TAT GAC CAT G-3'), 1.2μL Dye Terminator mix and 3.6μL of 5x buffer in a final volume of 12μL. Internal primers were also used for sequencing where appropriate. The reaction was thermocycled initially at 96°C for 1 min, then 25 cycles of 96°C for 10sec, 50°C for 5sec and 60°C for 4min. The sequencing reaction was then precipitated in 72μL of fresh 70% isopropanol at room temp for 15min, followed by centrifugation at 16,000g for 30min at 4°C with a further wash with 400μL of 70% isopropanol. The pellet was dried via centrifugation under vacuum for 5min and sent for sequencing. Sequence results were then assembled and analysed using BioEdit software (Isis Pharmaceuticals Inc., Carlsbad, California). For phylogenetic analyses, the predicted protein sequences were aligned using ClustalW, subjected to Maximum Likelikood

analysis using MolPhy version 2.3 via the Dayhoff model and in local rearrangement of NJ trees mode (1000 bootstrap replicates) (Adachi and Hasegawa, 1996). In these analyses, platypus (*Ornithorhynchus anatinus*, AAG00453) and human (*Homo sapiens*, CAI41386) factor X sequences were defined as outgroups.

<u>Identification of a Non-enzymatic Factor V-like Gene</u>

PCR amplification of the full-length cDNA transcript for the non-enzymatic factor V-like unit of the snake prothrombin activating complex was performed in group C elapids using primer sequences designed from the previously published *P. textilis* sequence (AY168281). The complete mRNA transcript of 4.4kb was amplified as two overlapping PCR fragments of 2271bp (fragment 1, including start codon) and 2202bp (fragment 2, including stop codon). The PCR reaction mix was made to final volume of 25μL containing the following: approximately 200ng of cDNA template, 1unit of AmpliTaq gold buffered with 10mM Tris-HCl pH 8.3, 50mM KCl, 2mM MgCl$_2$, 200μM dNTPs and 50ρmol of each of the forward and reverse primer. All reactions were run with appropriate no template controls and cDNA was examined from all eight elapids involved in the study. Fragment 1 was amplified with forward (5'- ATG GAA GAT ACA GTG TGA GCC C-3') and reverse primer (5'-CTC TGT CTG TTC CCT GCG TGG C-3') while fragment 2 was amplified with forward (5'-CCA GAC CCA GAA TCG GAT GCG C-3') and reverse primer (5'-GCC TTA AAA AAC TTC ACA ACC-3'). Both reaction mixtures were then thermocycled for 95°C for 10min, followed by 37 cycles of 95°C for 30sec, 64°C for 45sec (fragment 1) or 59°C for 45sec (fragment 2) and 72°C for 2min 30sec, followed by a final extension of 72°C for 7min.

Both PCR products were visualised on a 1.25% TAE agarose gel stained with ethidium bromide and the appropriate band excised and purified from *P. textilis*, *O. scutellatus* and *O. microlepidotus* with a QIAex II Gel extraction kit as previously described. Excised products were then ligated into pGEM-T vector system, transformed into dH5α *E. coli* cells, plasmid purified with a QIAprep Spin Miniprep kit. Multiple clones from each snake were then sequenced as previously described and alignments performed. Due to the large size of insert in the plasmid, primers internal to the factor V-like fragments were used in sequencing. These primers included sequences designed from *P. textilis* at positions 601 (5'-CTG AAT GCA AAT GGT TC-3'), 770 (5'-GCT GGC ATT TGA TAG GAA TG-3'), 1197 (5'-CAG

GCA ATA TGA AGA CGG A-3'), 2767 (5'GCT ATG ACG ACA AGT CTC C-3'), 3003 (5'-CAG GAC AAT AGA CAT AAG GG-3'), 3313 (5'-CTT CCT GGT ACA TTC GCC-3') and 3836 (5'-GAC TGC ATC CAA CCA AG-3') of the mRNA transcript.

Subsequent to identifying the full-length cDNA sequence of the factor V-like gene in group C elapids as two fragments, the entire transcript was amplified as a single product using the Expand Long Template PCR System (Roche Diagnostics, Basel, Switzerland). A 25μL reaction was prepared with approximately 200ng of cDNA from all snakes as a template, amplifying with 2 units of Expand Long Template Enzyme mix in PCR buffer 1 preheated to 50°C for 5min with 1.75mM $MgCl_2$ plus 360mM dNTPs. A single product was amplified using a forward primer corresponding to the 5'-UTR sequence of *P. textilis* (5'-CAG GCA GAA CTG ACT TCC GTG-3') and the same reverse primer originally used to amplify fragment 2 (5'-GCC TTA AAA AAC TTC ACA ACC-3'). 12μL of PCR product was run on a 0.8% TAE agarose gel and visualised with ethidium bromide, however cloning was not performed.

Quantitative PCR Analysis

Transcription of the factor X- and factor V-like prothrombin activator genes were analysed via quantitative PCR (qPCR). cDNA was synthesised from 1.5μg of venom gland RNA for each snake, as well as liver RNA purified from *O. scutellatus*. Prior to cDNA synthesis, total RNA was digested with 2 units of RQ1 DNAse-polymerase to remove any contaminating genomic DNA that may affect expression results (Promega, Madison, Wisconsin).   DNAse treated RNA was ethanol precipitated prior to cDNA synthesis and 1:50 dilution of the cDNA then used in subsequent qPCR reactions as template. In all cases 7.5μL of a 2x Sybr Green PCR master mix was added to 5ρmol of a forward and reverse primer mix and 3μL of diluted cDNA template in a total volume of 15μL (Applied Biosystems, Foster City, California). A 190bp fragment of the factor X-like protease was amplified with forward (5'-GAA GAC CCC TAT CCA GTT CTC-3') and reverse (5'-CAT GCA GGT GTG CCT GTC CAC A-3') primer pair whilst a 201bp section of the factor V-like transcript was amplified with forward (5'-CTC AAG GAG CCA CAT CAA TGA C-3') and reverse (5'-GGA TGA TAC GAA TGA ACC TGG AC-3') primers, based on sequence 100% identical to all snakes. Each template was run in triplicate and every run included the amplification of a 60S ribosomal

protein house-keeping gene, which was amplified as a 195bp product with forward (5'-GCA AGC GTA TGA ACA CCA ACC C-3') and reverse (5'-AGA GCA GCT GGG ACG ACC ATT C-3') primers.

Reaction mixtures were thermocycled at 95°C for 10min, followed by 35 cycles of 95°C for 15sec, 64°C for 15sec and 72°C for 30sec on a Rotor-Gene 3000 (Corbett Research, Mortlake, Australia). A standard curve of cycle time using a range of known quantities of plasmid as template was also performed. PCR products were run on a 1% TAE agarose gel to confirm amplification and the results including cycle time for product formation and calculated transcript concentration as determined by the Rotor-Gene software exported into Microsoft Excel. Averages of the triplicate samples in duplicate runs were then taken, and the results normalised to that of the house-keeping gene.

Prothrombin Activator Detection in Venom

The presence and quantity of the factor X- and factor V-like components of the snake prothrombinase complex were examined in the venom of all snakes by immunoblotting with a number of antibodies. For all assays, a total of 10μg of lyophilised venom resuspended in 50% glycerol from *O. scutellatus*, *O. microlepidotus*, *P. textilis*, *N. scutatus*, *T. carinatus*, *H. stephensii*, *P. porphyriacus* and *P. australis* were loaded onto an SDS-polyacrylamide gel in that order, with an appropriate pre-stained ladder (Fermentas, Hanover, Maryland). All gels were also run with approximately 25ng of purified native *P. textilis* factor Xa-like protease as a positive control. Initially a 12% SDS-PAGE was performed under reducing conditions and stained with colloidal coomassie to ensure approximate even loading of venoms and to visualise the variation in size and abundance of all venom components.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Coomassie Staining

Tris-Glycine SDS polyacrylamide gels were prepared and run with a mini-protean electrophoresis system (Biorad, Hercules, California). Gels were prepared with either a 12% (12% acrylamide mix, 375mM Tris pH 8.8, 0.1% SDS, 0.1% ammonium persulphate and 0.04% tetramethylehtylenediamine (TEMED)) or 15% separating phase and a 5% stacking phase (5% acrylamide mix, 125mM Tris pH 6.8, 0.1% SDS, 0.1% ammonium persulphate

and 0.04% TEMED). Protein samples were then run under either native conditions with 3μL of sample buffer (250mM Tris-HCl pH 6.8, 4.5% SDS, 20% glycerol and 0.025 w/v bromophenol blue) or under reducing conditions, whereby they were incubated with 3μL of sample buffer containing 5% β-mercaptoethanol at 100°C for 5min. Samples loaded on SDS-PAGE were then run at a constant current of 15mA per gel in running buffer (100mM Tris, 384mM glycine and 0.2% SDS). All samples were run against 10μL of a pre-stained molecular weight protein marker (New England Biolabs, Beverly, Massachusetts).

Total protein loaded on a SDS-PAGE was then examined by staining the gel with colloidal coomassie (0.1% w/v coomassie brilliant blue G-250, 2% w/v phosphoric acid, 10% ammonium sulphate and 20% methanol) shaking at room temperature overnight (Amresco, Solon, Ohio). The gel was washed briefly with water the following morning and fixed with drying film prior to scanning and storage as a digital image.

Immunoblot Analysis of Venom Proteins

Venoms run on a 12% SDS-PAGE under either reducing or non-reducing conditions were transferred to nitrocellulose membrane for immunoblot analysis with a range of primary antibodies. SDS-PAGE gels were placed in contact with a Hybond C nitrocellulose membrane (Amersham Biosciences, Cardiff, United Kingdom) and protein transferred in a mini-protean rig with high molecular weight transfer buffer (100mM Tris, 40mM glycine, 0.036% SDS and 20% methanol) at 100V for 1hour at 4°C. The membrane was then washed briefly in PBS/T buffer (with 0.05% Tween 20) before blocking with blotto (5% w/v skim milk powder in PBS/T) for 1hour at room temperature.

Membranes containing transferred venom proteins were then probed with a suite of specific antibodies. These included a primary antibody raised in sheep against a recombinant form of the factor X-like heavy chain from *P. textilis* (residues D302→L467), diluted in 1:5,000 in blotto and hybridised to the membrane at 4°C overnight. This antibody was previously prepared by Dr Igor Filippovich and Dr Natasha Sorokina from the Queensland Institute of Medical Research. The membrane was then subjected to three 5min washes in PBS/T the following morning followed by hybridisation with a Horse Radish Peroxidase (HRP)-conjugated sheep anti-mouse secondary antibody diluted 1:10,000 in blotto, shaking at room

temperature for 1hour (Chemicon, Melbourne, Australia). After a further three 15min washes in PBS/T, the membrane was exposed to a freshly prepared 1:1 ECL mixture (western lightning enhanced chemiluminescence luminal reagent and oxidising reagent, Perkin-Elmer, Norwalk, Connecticut). The blot was then exposed to Fuji medical X-ray film for increasing amounts of time and the developed results scanned as a digital image.

The heavy chain of the snake proteases were similarly detected by performing a 12% SDS-PAGE under reducing conditions and immunoblotting with the same antibody. This blot was then stripped of antibody by incubating the membrane in stripping buffer (125mM Tris pH 7.5, 2% SDS and 0.1% v/v β-mercaptoethanol) at 50°C for 45min, washed with PBS/T and re-blocked with blotto. A 1:500 dilution of primary antibody raised in rabbit against the complete native prothrombin activator complex from *P. textilis* was then hybridised to the membrane, followed by probing with a 1:5,000 dilution of anti-rabbit secondary antibody. Digital scans of the resulting blots were then performed following ECL detection. Finally a similar 12% SDS-PAGE was run under reducing conditions and the subsequent blot probed with a commercially available monoclonal antibody raised against human γ-carboxyglutamyl (Gla) residues. Protein was then detected by immunoblotting with a 1:5,000 dilution of HRP-conjugated anti-mouse secondary antibody.

Cloning of *O. scutellatus* Factor X-like Protease into pMIB/V5 HisA

Two different constructs of the factor X-like protease isolated and cloned from the venom gland of *O. scutellatus* were cloned into the vector pMIB/V5 HisA for recombinant protein expression, purification and characterisation. The two constructs generated included the full length precursor protease with propeptide, light and heavy chains linked by the activation peptide (given the name Pre-Prot), whilst the second construct had the propeptide sequence deleted (Mat-Prot). The pMIB/V5 HisA vector is a 3.6kb insect cell expression vector whose secreted product includes a cleavable Honey Bee Melittin secretion signal for export of the recombinant protein out of the cell and into the growth media, a C-terminal V5 epitope for protein identification by immunoblot analysis and a 6xHis tag for protein purification (Invitrogen, Mt Waverly, Australia).

Cloning of both constructs into the pMIB/V5 HisA multiple cloning site was performed with the incorporation of Hind III and Xba I cleavage sites at the 5' and 3' ends respectively of the

previously cloned protease transcript via PCR. A 50μL reaction was prepared with approximately 50ng of *O. scutellatus* protease in pGEM-T vector as template for both constructs, 2 units of AmpliTaq gold buffered in 10mM Tris-HCl pH 8.3, 50mM KCl and 1.5mM $MgCl_2$ with 200μM dNTPs and 50ρmol of each of the forward and reverse primer. The reverse primer with Xba I restriction site was consistent for both constructs (5'-GCT CTA GAG AGC CGA CCA GTG CTT GAC TCT GT-3') however Pre-Prot was amplified with forward primer (5'-CCC AAG CTT GAT GGC TCC TCA ACT ACT CCT CTG T-5') whilst Mat-Prot was amplified with forward primer (5'-CCC AAG CTT GAA TTC ACT GTA TGA GGA ATT TAG ATC TGG-3'). The reactions were thermocycled initially at 95°C for 10min, then 27°C cycles of 95°C for 30sec, 60°C for 40sec and 72°C for 1min 30sec with a final extension of 72°C for 7min.

A 3μL aliquot of the PCR product was run on a 1% TAE agarose gel to confirm its amplification. The remaining PCR product was ethanol precipitated as previously described and the pellet resuspended in 14μL of water. The PCR product was double digested with 20 units of Hind III and 20 units of Xba I buffered in 10mM Tris-HCl pH 7.9, 50mM NaCl, 10mM $MgCl_2$, 1mM dithiothreitol plus 100ng BSA in a final volume of 20μL at 37°C for 2hours (New England Biolabs, Beverly, Massachusetts). A similar double restriction digest was also performed on approximately 3μg of pMIB/V5 HisA vector, as well as single digests with each of the restriction enzymes to confirm cleavage with both. The total product for each was run on 1% TAE agarose gel stained with ethidium bromide and the digested bands excised and purified with a QIAex II Gel extraction kit as previously described. The concentraion of the purified product was then measured at an absorbance of 260nm.

The double digested and purified PCR products for both constructs were then cloned in frame into the double digested pMIB/V5 HisA plasmid by use of their matching 5' and 3' overhanging sequences. Ligation was performed with 7 units of T4 DNA Ligase in the presence of 30mM Tris-HCl pH 7.8, 10mM $MgCl_2$, 10mM DTT and 1mM ATP 5% PEG in a final volume of 20μL at 16°C overnight. Insert to vector ratio was approximately 2:1. The ligation mixture was ethanol precipitated the following morning and used to transform electrocompetent dH5α *E. coli* cells as previously described. Multiple clones were selected and purified with a QIAprep spin miniprep kit and the presence of inserts confirmed by a double restriction digest with Hind III and Xba I as described above. Clones positive for

inserts were then sequenced in both the forward (5'-CGC AAC GAT CTG GTA AAC AC-3')
and reverse (5'-GAC AAT ACA AAC TAA GAT TTA GTC AG-3') direction with primers
specific for the pMIB/V5 HisA vector, to ensure the correct constructs had been cloned in
frame prior to transfection and expression.

Plasmid Midiprep

Larger scale preparations of plasmid were performed prior to transfection and expression with
a Perfectprep® Plasmid Midi kit according to manufacturer's instructions (Eppendorf,
Hamburg, Germany). A 50mL culture of transformed dH5α containing the desired construct
from above was grown in LB broth with 100μg/mL ampicillin at 37°C overnight. The cells
were pelleted by centrifugation at 3,000g for 10min at 4°C, resuspended in cold solution 1
then incubated at room temperature for 10min and ice for 5min. A further 4mL of solution 2
was added and the mixture incubated on ice for 5min, followed by 4mL of solution 3 with
gentle mixing and 10min incubation on ice. The preparation was then centrifuged at 5,000g
for 15min and the supernatant removed to a fresh tube where 10mL of cold DNA-binding
matrix was added and mixed by vigorous inversion. The entire mixture was then loaded on a
provided spin column and centrifuged at 2,000g for 5min at room temperature. 10mL of
diluted purification solution was then added to the column and centrifuged at 2,000g for 5min.
The plasmid DNA was then eluted via the addition of 1mL of elution buffer and recovered by
centrifugation at 2,000g for 5min. The purified plasmid constructs were then quantified by
measuring absorbance at 260nm and its quality confirmed by running on a 1% TAE agarose
gel stained with ethidium bromide.

Recombinant Protease Transfection and Expression

Transfection and Cell Maintenance

The recombinant *O. scutellatus* protease constructs were transfected into SF9 insect cells with
Gene Jammer lipid transfection reagent as follows (Stratagene, La Jolla, California). A 6 well
plate was seeded with $6x10^5$ mid-log phase SF9 cells/well that were then allowed to grow in
2mL Ex-Cell 420 serum free media at 27°C overnight (JRH Biosciences, Lenexa, Kansas). A
mixture of 100μL of media and 6μL Gene Jammer transfection reagent was then allowed to

incubate at room temperature for 10min before 1μg of the desired plasmid (that is, either Pre-prot or Mat-prot in pMIB/V5 HisA) was added followed by a further incubation of 10min. Note that all tissue culture procedures were performed in a sterile laminar flow hood. The media in the wells was then replaced with 900μL of fresh media, followed immediately by the addition of the above preparation of transfection reagent and plasmid. Appropriate controls containing Gene Jammer only, plasmid only and no Gene Jammer or plasmid were also prepared. The cells were then incubated at 27°C for 4hours with intermittent rocking. A further 1mL of serum free media containing 0.5% antibiotic/antimycotic was added to each well (Invitrogen, Mt Waverly, Australia).

The cells were then allowed to grow until they obtained 80-100% confluency in monolayer (approximately 24hours) whereupon they were split 1:2. Blastocidine selection reagent (Invitrogen, Mt Waverly, Australia) at a final concentration of 25μg/mL was added to one well and SF9 cells transfected with the desired pMIB/v5 HisA construct allowed to grow at 27°C. Cell cultures were then passaged in a 6 well plate monolayer a further two times so as to obtain fully selected cells prior to increasing the culture volume to 25cm^2, 75cm^2 and eventually 150cm^2 sized ventilated flasks, never allowing the confluency level to drop below 20%. Blastocidine selected cells expressing the construct were then taken up to a 100mL suspension culture containing 0.5% antibiotic/antimycotic and 10μg/mL blastocidine, and grown shaking at 135rpm at 27°C. Cell counts were performed by diluting an aliquot of the suspension culture 1:4 with 0.04% Trypan blue in Dulbecco's solution and counting viable cells with a haemocytometer. Suspension cultures containing vitamin K were seeded with 5x10^5 cells/mL for optimal growth rates, with regular passaging (SF9 cells have a doubling time of 24 hours and will not grow above 1x10^7 cells/mL). Expressed recombinant protein could then be recovered by centrifuging the suspension culture at 3,500g for 8min and storing the supernatant.

Cryopreservation and Cryoresurrection

Healthy mid-log phase SF9 cells expressing either the Pre-prot or Mat-prot constructs were prepared for long term storage in liquid nitrogen using the following cryopreservation protocol. Cells were pelleted by a gentle centrifugation at 163g for 5min with slow brake and resuspended in the old media so as to give a concentration of 2x10^7 cells/mL. An equal

volume of fresh media containing 15% DMSO was also prepared and added to the old media on ice (bringing the final concentration of DMSO to 7.5%). 1mL aliquots of the culture containing $1x10^7$ cells/mL were then frozen to -70°C overnight in a controlled cooling device (Nalgene, Rochester, New York State) whereupon they were transferred to liquid nitrogen storage at -150°C. A single aliquot of frozen cells were then resurrected to check their viability. A partially thawed preparation was added to 9mL of fresh Ex-Cell 420 media and centrifuged at 163g for 5min with slow brake to pellet the cells. The supernatant was then discarded (to remove any DMSO), the pellet resuspended in a further 15mL of fresh media and cells grown in a 75cm^2 monolayer flask at 27°C. The culture was then checked the following day to ensure the cryoresurrected cells were capable of growth.

Recombinant Protease Purification and Detection

Nickel Column Purification

The expressed recombinant His-tagged protease constructs secreted into supernatant were purified via nickel column chromatography. A total of 1L of either the Pre-prot or Mat-prot suspension culture were grown for at least 24hours after the concentration of cells reached $1x10^7$ cells/mL. The cells were then pelleted via centrifugation at 3,500g for 8min and the supernatant recovered. Protein in the media was buffer exchanged to one more optimal for purification on a nickel column by first concentrating the supernatant to 200mL by applying reverse pressure whilst pumping the solution through a closed circuit with an outlet covered by a 10kDa filter (Pall, East Hills, New York State). Buffers were then exchanged keeping a constant volume (thereby minimising protein precipitation), again under reverse pressure in a closed system. Two different binding buffers for exchange of the protein out of the media were experimented with: the first was a phosphate buffer containing 50mM NaH_2PO_4 pH 8.0, 300mM NaCl, 10mM imidazole and the second was a Tris buffer containing 20mM Tris-HCl pH 7.5, 300mM NaCl, 5mM imidazole. These binding buffers served as the basis for future washing or elution buffers.

His-tagged recombinant protein was then purified on the basis of the QIAexpress Ni-NTA protein purification system (Qiagen, Hilden, Germany). 2mL of mixed Ni-NTA agarose was loaded onto a chromatography column and allowed to pack down, giving a final volume of

1mL. All subsequent steps were performed at 4°C and the column was never allowed to dry out. A total of 5mL of the relevant binding buffer was run through the column, followed by loading of the 200mL sample in binding buffer at a rate of 1mL/min. Following loading of the sample, four washes of the column were performed with increasing imidazole concentrations (a competitive binder of nickel): wash 1 was 10mL of binding buffer, wash 2 was 10mL of binding buffer with 20mM imidazole, wash 3 was 10mL of binding buffer with 40mM imidazole and wash 4 was 10mL of binding buffer with 60mM imidazole. His-tagged protein was then eluted with four 1.5mL fractions of elution buffer (binding buffer containing 250mM imidazole) and stored at 4°C. The column was then stripped of nickel with 10mL of stripping buffer (20mM Tris-HCl pH 7.9, 100mM EDTA and 500mM NaCl) and stored in the same buffer. Prior to reuse the column was washed with 10mL of sterile water and reloaded with nickel by loading 5mL of 50mM NiSO$_4$. In this fashion a single column was used for protein purification up to four times.

1mL each of eluted fractions 1 and 2 of recombinant protease were combined and dialysed against 1.5L of 10mM Tris-HCl pH 7.4 and 150mM NaCl to remove imidazole with a 3kDa Slide-A-Lyzer dialysis cassette at 4°C overnight (Progen Biosciences, Archerfield, Australia). 500μL of the purified sample was then added 1:1 to 80% glycerol and stored at -20°C, whilst the remaining 1.5mL was stored at 4°C. Purified fractions of recombinant protein were then analysed via SDS polyacrylamide gel electrophoresis and subsequent colloidal coomassie or western blot analysis as described below.

Protein Quantitation: Lowry Assay

The concentration of total protein in eluted fractions of purified recombinant protease was estimated via the Lowry Assay according to manufacturer's instructions (Biorad, Hercules, California). A protein standard curve was established by preparation of BSA in PBS buffer ranging in concentrations from 200μg/mL to 800μg/mL and run in conjunction with the purified samples.   10μL all samples and standards were loaded in triplicate in a microwell format. To each well 25μL of Reagent A' was added (prepared by adding 20μL of reagent S to 1mL of reagent A), followed by 200μL of reagent B. After a brief incubation, absorbance was read at 750nm with a microwell plate reader and protein concentration automatically determined for the average of the triplicates by reference to the BSA standard curve.

Russell's Viper Venom Activation

Recombinant protease construct samples, including culture supernatant as well as the nickel purified His-tagged protein described above, were also analysed after activation with Russell's Viper Venom (RVV). Reaction mixtures containing RVV activation buffer (at a final concentration of 10mM Tris-HCl pH 7.4, 150mM NaCl, 10mM $CaCl_2$ and 0.02% Tween 20), 125ng/μL RVV and appropriate protein sample were incubated at 37°C for approximately 90min prior to testing activity and analysing on SDS polyacrylamide gels.

Immunoblot Analysis of Recombinant Proteins

Native and reduced Pre-prot and Mat-prot protease constructs secreted into the insect cell culture supernatant (with and without RVV activation), as well as wash and elution fractions from the nickel column purification were detected with a commercial preparation of mouse anti-V5 antibody diluted in 1:5,000 (Invitrogen, Mt Waverly, Australia). The membrane was then probed with a 1:5000 dilution of anti-mouse secondary antibody and signal detected as previously described. Detection of the His-tag purified recombinant protease (with and without RVV activation) was also performed with a 1:500 dilution of primary antibody against native *P. textilis* prothrombin activator complex raised in sheep. Western blots were also performed with a 1:1 mixture of primary antibodies raised in sheep against recombinant *P. textilis* protease light chain (amino acid residues 59-188) and heavy chain (amino acid residues 302-467) diluted 1:500 and detected with ECL after 1:5,000 rabbit anti-sheep secondary antibody hybridisation. Finally, western blot analysis was also performed with the aforementioned commercially available monoclonal antibody raised against human γ-carboxyglutamyl (Gla) residues, a post-translational modification observed on active native snake prothrombin activators.

Recombinant Protease Activity Assays

Chromogenic Assay

Activity of the Pre-prot and Mat-prot recombinant *O. scutellatus* protease constructs was initially determined via chromogenic assay with a prothrombin-like substrate specifically cleaved by factor Xa-like molecules linked to p-nitroaniline. This chromophoric group is

released upon active cleavage of the substrate and may be measured photometrically at an absorbance of 405nm. Activity of recombinant protease was tested with and without activation by Russell's Viper Venom of not only purified fractions of the protease but also on that found in the culture supernatant. The reaction was performed in 1mL with either 500μL of supernatant or 100μL of purified protein, 75μM S-2222 or S-2765 chromogenic substrate buffered in RVV activation buffer (final concentration of 10mM Tris-HCl pH .74, 150mM NaCl, 10mM $CaCl_2$ and 0.02% Tween 20) (Chromogenix, Milano, Italy). Absorbance was read every 5sec for 3min and the rate of activity calculated. Positive controls were run with 4μg of native *P. textilis* protease, as well as negative controls with RVV only, untransfected insect cell culture supernatant, Ex-Cell 420 media only and water only blanks.

Coagulation Assays

A whole blood clotting assay was also performed with a Thromboelastograph® 5000 Haemostasis Analyser which measures the time and rate of clot formation in a citrated blood sample (Haemoscope Corporation, Niles, Illinois). Pooled citrated whole blood samples supplied by the Princess Alexandra Hospital were examined for the time and rate of clot formation upon the addition of 20μL of 200mM $CaCl_2$ (that is, recalcification) in a final volume of 360μL. 50μL of purified fractions of both Pre-prot and Mat-prot were added with 20μL of 200mM $CaCl_2$ and 290μL of whole blood in duplicate runs, and the time for clot formation calculated as a percentage of the standard time. Experiments were also repeated with 100μL of recombinant protease plus $CaCl_2$ in a final volume of 360μL. Appropriate negative controls including Ex-Cell 420 media only and untransfected SF9 cell supernatant only were also performed, and all experiments were carried out at room temperature. Activity of the recombinant protease after activation with RVV could not be tested, as RVV is in itself highly procoagulant.

Protease Mutagenesis

A mutagenic construct of the full-length *O. scutellatus* prothrombin activator (designated Pre-Mut) was then prepared and analysed in a fashion similar to that of the previous two constructs. Two amino acids before, and four amino acids after the cleavage site between the light chain and the activation peptide were mutated to directly reflect those of the cleavage

site between the heavy chain and the activation. That is, the sequence RNKREA (5'-AGG AAC AAG AGG GAA GCA-3') was mutated to PDIRIV (5'-CCG GAC ATC AGG ATA GTA-3') via the incorporation of the new sequence with complementary primers, and PCR amplification of the entire vector, followed by its recircularisation and purification.

PCR amplification of the vector with mutated primers was performed with 65ng, 130ng and 195ng of Pre-Prot plasmid as template in a 50μL reaction. The reaction contained 3.75 units of Pfu Turbo polymerase (Stratagene, La Jolla, California) buffered in 20mM Tris-HCl pH 8.8, 2mM MgSO$_4$, 10mM KCl, 10mM (NH$_4$)$_2$SO$_4$, 0.1% Triton X-100 and 100μg/mL BSA with 300μM dNTPs and 125ng each of the forward (5'-GTG GTA CAA ATA TCA AAA CAC CGG ACA TCA GGA TAG TAA GTC TGC CTG ACT TTG TGC-3') and reverse (5'-GCA CAA AGT CAG GCA GAC TTA CTA TCC TGA TGT CCG GTG TTT TGA TAT TTG TAC CAC-3') primers. The reaction mixture was then thermocycled at 95°C for 2min, followed by 17 cycles of 95°C for 40sec, 64°C for 50sec and 68°C for 8min. 4μL of PCR product was run on a 1% TAE agarose gel to confirm amplification and the remainder digested with 30 units of Dpn1 restriction enzyme (buffered in 20mM Tris-acetate pH 7.9, 50mM potassium acetate, 10mM magnesium acetate and 1mM DTT) at 37°C to remove the original vector template which is bacterial in origin and therefore methylated and susceptible to Dpn1 cleavage (New England Biolabs, Beverly, Massachusetts).

The restriction digest mix was ethanol precipitated as previously described, resuspended in 12μL of sterile water, 1μL of which was run on a 1% TAE agarose gel to check the quality of the DNA. 5μL was then transformed into electrocompetent dH5α, plated on LB agar with ampicillin and grown at 37°C overnight (the *E. coli* cells recircularise the vector care of the overlapping and complementary primer sites). Colonies were selected for miniprep purification of plasmid and checked for the presence of inserts by restriction digest with Hind III and Xba1 restriction enzymes as previously described. Positive clones were then sequenced to confirm the incorporation of the mutated site. The Pre-mut mutated protease construct was then transfected into SF9 insect cells, expressed, purified, detected and characterised via the methods described above for the other constructs.

Results

RNA Isolation from Australian Elapids

RNA was isolated successfully from the venom glands of all 8 Australian elapids involved in the study. Figure 2.01B demonstrates the quality and quantity of RNA recovered from the venom gland of *O. scutellatus* (note the presence of ribosomal bands, in addition to the high molecular weight transcripts). The total yield of RNA per pair of venom glands ranged from 55μg to 578μg between snakes. This variation in amounts reflects the size of the glands per snake and not the concentration of RNA within any given venom gland.
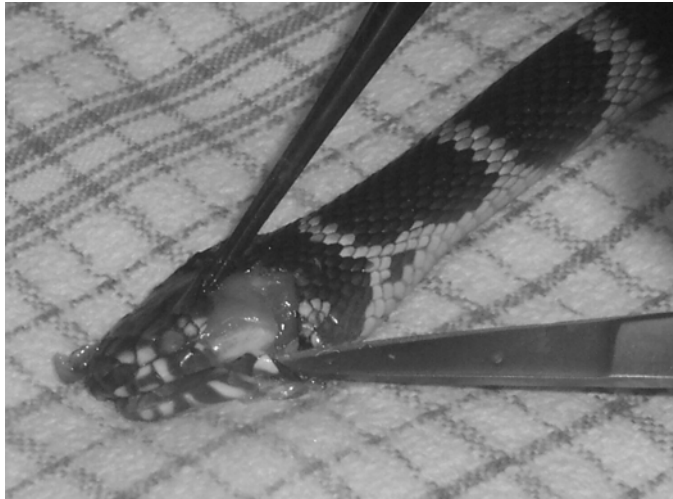
A.   **B.**
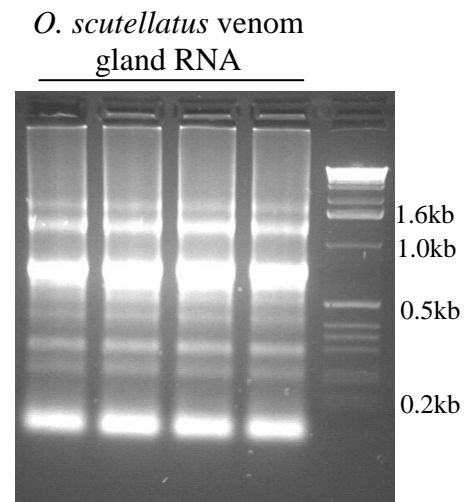
Figure 2.01. Venom gland RNA preparation.
(A) Dissection of a venom gland from the Stephen's banded snake, *H. stephensii*. **(B)** Total RNA isolated from the venom gland of the coastal taipan, *O. scutellatus*.

Identification of Factor X-like Protease cDNAs from Australian Elapids

Full-length cDNA clones corresponding to the coding sequence of the factor X-like prothrombin activator were isolated from the venom glands of Australian elapids based on the previously identified *P. textili*s protease gene sequence (Filippovich *et al.*, 2005; Rao *et al.*, 2004). Two primers designed from the propeptide and heavy chain gene sequences were used to amplify an approximately 1.4kb fragment. The results in figure 2.02 demonstrate the

presence of a PCR product in *P. textilis*, *O. scutellatus*, *P. porphyriacus*, *N. scutatus*, *T. carinatus* and *O. microlepidotus*. A similar product was also amplified from the venom gland cDNA of *H. stephensii* (results not shown), however, protease mRNA was not detected in the mulga (*P. australis*) even when additional primer pairs, based on the internal gene sequence of *P. textilis*, were employed. This is not surprising given that the venom of this snake does not demonstrate procoagulant activity (Dambisya *et al.*, 1995; Tan and Ponnudurai, 1990).
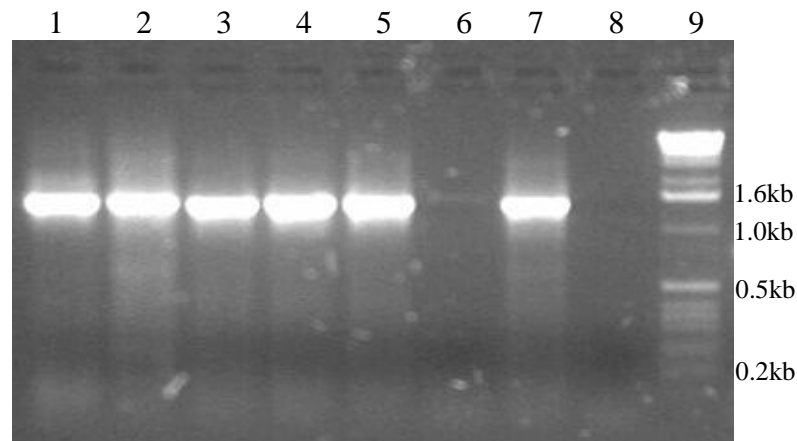
Figure 2.02. PCR of the factor X-like cDNA.

PCR amplification of the complete 1.4kb factor X-like protease transcript from venom gland cDNA isolated from 1) *P. textilis*, 2) *O. scutellatus*, 3) *P. porphyriacus*, 4) *N. scutatus*, 5) *T. carinatus*, 6) *P. australis*, 7) *O. microlepidotus*, 8) no template control and 9) molecular weight marker. A similar product was also amplified from the venom gland cDNA of *H. stephensii* (not shown).

Multiple clones were generated to identify the protease cDNA sequence in each snake for nucleotide comparisons. The overall identity at the nucleotide level was 82.5% when an alignment was performed between all seven snake species. A protein alignment of the predicted amino acid sequence from these cDNAs is depicted in figure 2.03. The overall identity between the predicted snake proteins is 71.5%. Comparison of the two taipan species, *O. scutellatus* and *O. microlepidotus*, reveals an identity of 95% (table 2.01). These proteases were most closely related to that of *P. textilis* with a three-way identity of 91%. Whilst the degree of homology between all snake species was high (80-97%), the protease from *P. porphyriacus* was least related to the others.

```
                    ┌─→ Propeptide                          ┌─→ Light chain
                         10        20        30             ↓↓      50   ↓    ↓↓       ↓↓      ↓   70 ↓↓    ↓  80
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
O. scutellatus     1  MAPQLLLCLILTFLWSLPEAESNVFLKSKVANRFLQRTKRANSLYEEFRSGNIERECIEERCSKEEAREVFEDDEKTETF  80
O. microlepidotus  1  MAPQLLLCLILTFLWSLPEAESNVFLKSKVANRFLQRTKRANSLFEEFRSGNIERECIEERCSKEEAREVFEDDEKTETF  80
P. textilis        1  MAPQLLLCLILTFLWSLPEAESNVFLKSKVANRFLQRTKRANSLVEEFKSGNIERECIEERCSKEEAREVFEDDEKTETF  80
P. porphyriacus    1  MAPQLLLCLILTFLWSLPEAESNVFLKSKEANRFLQRTKRSNSLFEEFRPGNIERECIEEKCSKEEAREIFKDNEKTEAF  80
H. stephensii      1  MAPQLLLCLILTFLWSVPEAESNVFLKSKVANRFLQRTKRSNSLFEETRPGNIERECIEEKCSKEEAREVFEDNEKTETF  80
N. scutatus        1  MAPQLLLCLILTFLWSLPEAESNVFLKSKVANRFLQRTKRSNSLFEETRPGNIERECIEEKCSKEEAREVFEDNEKTETF  80
T. carinatus       1  MAPQLLLCLILTFLWSLPEAESNVFLKSKVANRFLQRTKRSNSLFEETRPGNIERECIEEKCSKEEAREVFEDNEKTETF  80

                            90 ┌─ O-linked glycosylation 120        130       140       150       160
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
O. scutellatus     81 WNVYVDGDQCSSNPCHYRGTCKDGIGSYTCTCLSGYEGKNCERVLYKSCRVDNGNCWHFCKPVQNDIQCSCAEGYLLGED  160
O. microlepidotus  81 WNVYVDGDQCSSNPCHYRGTCKDGIGSYTCTCLFGYEGKNCERVLYKSCRVDNGNCWHFCKPVQNDIQCSCAEGYLLGED  160
P. textilis        81 WNVYVDGDQCSSNPCHYRGICKDGIGSYTCTCLSGYEGKNCERVLYKSCRVDNGNCWHFCKSVQNDIQCSCAEGYLLGED  160
P. porphyriacus    81 WNVYVDGDQCSSNPCHYGGTCKDGIGSYTCTCLPNYEGKNCEHLLFKSCRFFNGNCWHFCKPVQNDTQCSCAESYRLGDD  160
H. stephensii      81 WNVYVDGDQCSSNPCHYHGTCKDGIGSYTCTCLPNYEGKNCEKVLFKSCRAFNGNCWHFCKRVQSETQCSCAESYRLGVD  160
N. scutatus        81 WNVYVDGDQCSSNPCHYRGTCKDGIGSYTCTCLPNYEGKNCEKVLFKSCRAFNGNCWHFCKRVQSETQCSCAESYLLGVD  160
T. carinatus       81 WNVYVDGDQCSSNPCHYRGTCKDGIGSYTCTCLPNYEGKNCEKVLYQSCRVDNGNCWHFCKRVQSETQCSCAESYRLGVD  160

                                          ┌─→ Activation peptide            ┌─→ Heavy chain
                         170       180        190       200        220       230       240
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
O. scutellatus    161 GHSCVAGNFSCGRNIKTRNKREASLPDFVQSQNAILLKKSDNPSPDIRIVNGMDCKLGECPWQAVLVDEKEDAFCGGTI  240
O. microlepidotus 161 GHSCVAGNFSCGRNIKTRNKREASLPDFVQSQNATLLKKSDNPSPDIRIVNGMDCKLGECPWQAVLVDEKEGVFCGGTI  240
P. textilis       161 GHSCVAGNFSCGRNIKTRNKREASLPDFVQSHNATLLKKSDNPSPDIRIVNGMDCKLGECPWQAALVDDKKGVFCGGTI  240
P. porphyriacus   161 GHSCVAEGDFSCGRNIKARNKREASLPDFVQSQNATLLKKSDNPSPDIRITNGMDCKLGECPWQAVLLDKEGDVFCGGTI  240
H. stephensii     161 GHSCVAEGDFSCGRNIKARNKREASLPDFVQSQNATLLKKSDNPSPDIRIVNGMDSKLGECPWQAVLINEKGEVFCGGTI  240
N. scutatus       161 GHSCVAEGDFSCGRNIKARNKREASLPDFVQSKATVLKKSDNPSPDIRIVNGMDCKLGECPWQAVLINEKGEVFCGGTI  240
T. carinatus      161 GHSCVAEGDFSCGRNIKARNKREASLPDFVQSKATLLKKSDNPSPDIRIVNGMDCKLGECPWQAVLINEKGEVFCGGTI  240

                         *   ┌─ N-linked glycosylation  280       290       300 *      320
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
O. scutellatus    241 LSPIYVLTAAHCINQTKMISVVVGEINISRKNPGRLLSVDKIYVHQKFVPPKKGYEFYEKFDLVSYDYDIAILQMKTPIQ  320
O. microlepidotus 241 LSPIYVLTAAHCINQTEKISVVVGEIDKSRVETGHLLSVDKIYVHKKFVPPKKGYKFYEKFDLVSYDYDIAIIQMKTPIQ  320
P. textilis       241 LSPIYVLTAAHCINETETISVVVGEIDRSRAETGPLLSVDKVYVHKKFVPPKKSQEFYEKFDLVSYDYDIAIIQMKTPIQ  320
P. porphyriacus   241 LSPIYVLTAAHCITQSKHISVVVGEIDISRKETRHLLSVDKAYVHTKFVLA------------TYDYDIAIIQLKTPIQ  307
H. stephensii     241 LSPIHVLTAAHCINQTKSVSVIVGEIDISRKETRRLLSVDKIYVHTKFVPPNY-YYGHQNFDRVAYDYDIAIIRMKTPIQ  319
N. scutatus       241 LSPIHVLTAAHCINQTKSVSVIVGEIDISRKETRRLLSVDKIYVHKKFVPPNS-YY--QNIDRFAYDYDIAIIRMKTPIQ  317
T. carinatus      241 LSPIHVLTAAHCINQTKSVSVIVGEIDISRKETRRLLSVDKIYVHTKFVPPNY-YVHQNFDRVAYDYDIAIIRMKTPIQ  319

                         330       340       350       360       370       380       390       400
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
O. scutellatus    321 FSENVVPACLPTADFANQVLMKQDFGIVSGFGRIFEKGPQSKTLKVLKVPYVDRHTCMLSSSESPITPTMFCAGYDTLPRD  400
O. microlepidotus 321 FSENVVPACLPTADFANQVLMKQDFGIISGFGRIFEKGPKSNTLKVLKVPYVDRHTCMVSSESPITPTMFCAGYDTLPRD  400
P. textilis       321 FSENVVPACLPTADFANQVLMKQDFGIVSGFGGIFERGPNSKTLKVLKVPYVDRHTCMLSSNEPITPTMFCAGYDTLPQD  400
P. porphyriacus   308 FSENVVPACLPTADFANQVLMKQDFGIISGFGHTRSGCQTSNTLKVVTIPYVDRHTCMLSSDFRITPNMFCAGYDTLPRD  387
H. stephensii     320 FSENVVPACLPTADFANEVLMKQDSGIVSGFGRIRFKEPTSNTLKVITVPYVDRHTCMLSSDFRITQNMFCAGYDTLPQD  399
N. scutatus       318 FSENVVPACLPTADFAKEVLMKQDSGIVSGFGRTQSIGYTSNTLKVITVPYVDRHTCMLSSNFRITQNMFCAGYDTLPQD  397
T. carinatus      320 FSENVVPACLPTADFANEVLMKQDSGIVSGFGRIQFKQPTSNTLKVITVPYVDRHTCMLSSDFRITQNMFCAGYDTLPQD  399

                         *   410       420       430       440       450       460
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
O. scutellatus    401 ACQGDSGGPHITAYRDTHFITGIVSWGEGCAQTGKYGVYTKVSKFILWIKRIMRQKLPSTESSTGRL  467 AY940204
O. microlepidotus 401 ACQGDSGGPHITAYRDTHFITGIVSWGEGCAKKGKYGIYTKVSKFILWIKRIMRQKLPSTESSTGRL  467 AY940205
P. textilis       401 ACQGDSGGPHITAYRDTHFITGIVSWGEGCARKGRYGIYTKLSKFIPWIKRIMRQKLPSTESSTGRL  467 AY631238
P. porphyriacus   388 ACQGDSGGPHITAYRDTHFITGIISWGEGCAKKGKYGVYTKVSNFIPWIKAVMRKHQPSTESSTGRL  454 AY940207
H. stephensii     400 AGEGDSGGPHITAYGDTHFITGIVSWGEGCARKGKYGVYTKVSFIPWIKKIMSLK-----------  455 AY940208
N. scutatus       398 ACQGDSGGPHITAYGDTHFVTGIISWGEGCARKGKYGVYTKVSNFIPWIKKIMSLK-----------  453 AY940206
T. carinatus      400 ACQGDSGGPHITAYRDTHFITGIISWGEGCARKGKYGVYTKVSKFIPWIKKIMSLK-----------  455 AY769963
```

Figure 2.03. Factor X-like protein alignment.

Deduced amino acid sequences predicted from multiple cDNA clones of the factor X-like protease isolated from the venom glands of seven Australian snakes. Genbank accession numbers are provided at the end of each sequence. Sequence identity is shown in black, gaps are inserted for optimal alignment and the predicted cleavage sites for the propeptide, light chain, activation peptide and heavy chain sequences are also marked. The sites for γ-carboxylation of glutamic acid residues are marked by an ↓ and active site residues with an *. Conserved cysteine residues involved in putative disulfide bond formation are shaded gray, and glycoslation sites shown.

	O. scutellatus (Oscutarin C)	O. microlepidotus (Omicarin C)	P. textilis (Pseutarin C)	P. porphyriacus (Porpharin D)	H. stephensii (Hopsarin D)	T. carinatus (Trocarin D)	N. scutatus (Notecarin D)
H. sapiens	48.8%	47.9%	47.9%	47.1%	46.7%	47.5%	47.6%
N. scutatus	80.3%	81.0%	79.9%	83.0%	94.7%	95.0%	
T. carinatus	82.7%	83.1%	81.4%	82.9%	96.9%		
H. stephensii	81.2%	83.1%	79.9%	82.6%			
P. porphyriacus	82.0%	83.8%	81.0%				
P. textilis	92.1%	93.4%					
O. microlepidotus	95.1%						

Table 2.01. Percentage identity at the amino acid level of the factor X-like proteases.
Comparisons are drawn between the Australian elapids and the human homolog. Names assigned to the prothrombin activator from each of the species are denoted in parentheses.

Phylogenetic analysis of the deduced amino acid sequences resolved the snake factor X-like prothrombin activators into two distinct clades, each with high bootstrap support (figure 2.04). It is interesting to note that this clustering, based on sequence relatedness, separates the elapids into two distinct groups which have previously been defined on the basis of their requirement for mammalian factor Va as a cofactor for activity. Group C prothrombin activators, including proteases isolated from members of the *Pseudonaja* and *Oxyuranus* genera, do not require mammalian coagulation factor Va for activity as they have their own factor Va-like molecule complexed to the factor Xa-like protease. On the other hand the group D prothrombin activators do have a requirement for mammalian factor Va. Although the phylogenetic analyses strongly support a close relationship between the venom group D sequences, relationships within the group C clade are not well supported, which explains the implied paraphyly of the genus *Oxyuranus*. The diverged nature of the *P. porphyriacus* sequence is also clear in the tree shown in figure 2.04, although the analyses strongly support its basal position within the venom group D clade.

Comparison of all snake protease sequences with that of human factor X reveal that they possess a similar overall domain arrangement, including a propeptide, light chain, activation peptide and heavy chain in that order (MacGillivray and Fung, 1989). In all cases the 40 amino acid propeptide sequence was highly conserved, differing by only a single residue in *P. porphyriacus* and *H. stephensii* (figure 2.03). This sequence contains a high content (60%) of hydrophobic residues consistent with its role as secretion signal/signal peptide, and the only

two substitutions observed also led to conservation of hydrophobic residues. It is evident from the domain arrangement that this precursor protein requires processing to produce the mature heavy and light chain cross-linked protease, similar to that of its mammalian counterpart (Di Scipio *et al.*, 1977). The cleavage site between the propeptide and light chain is highly conserved between all snake species being R↓ANS in group C proteases and R↓SNS in group D prothrombin activators. The cleavage sites at either end of the activation peptide in the precursor protease are absolutely conserved in all snake species, with the exception of a hydrophobic amino acid substitution in the case of *P. porphyriacus* (V211I).
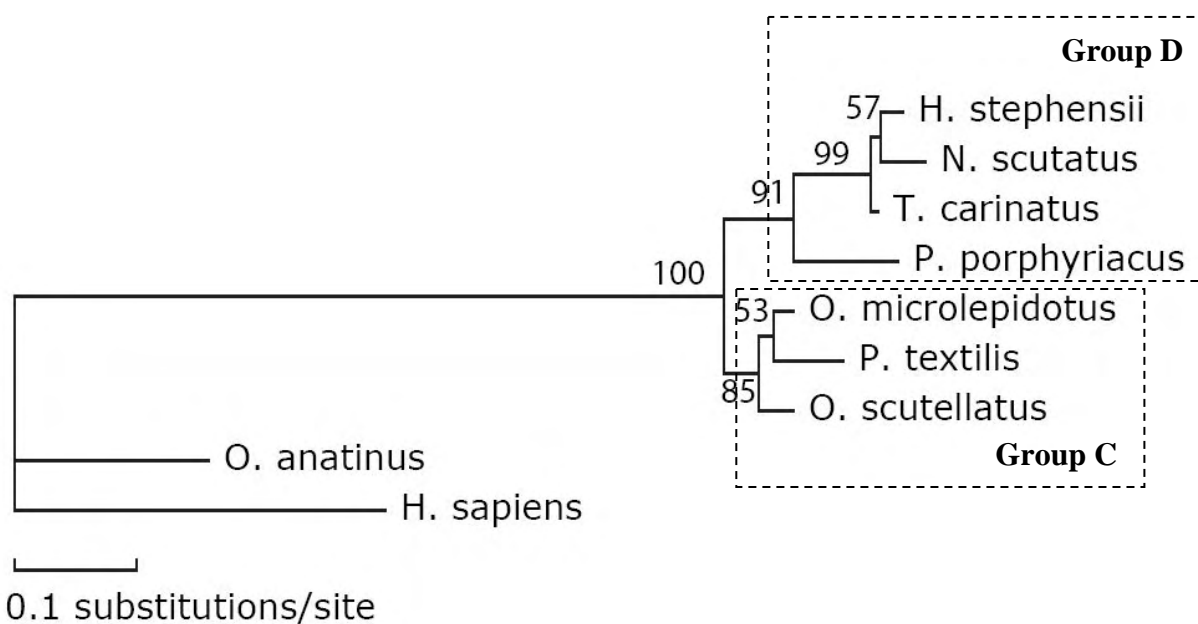
Figure 2.04. Phylogenetic relationship of the factor X-like proteases.
Analysis was performed from multiple Australian elapids using the Maximum Likelihood method on deduced amino acid sequences. Numbers above branches indicate the percentage of 1000 bootstrap replicates supporting the topology shown. Clades corresponding to group C and group D factor X-like proteases are clearly resolved with high bootstrap support.

While the protease sequences are highly conserved overall, the three most closely related snakes amongst the group D prothrombin activators (*H. stephensii*, *T. carinatus* and *N. scutatus*) have a premature stop codon that deletes the final 11 C-terminal amino acids compared to the group C proteases and the *P. porphyriacus* sequence. In addition, deletions of a variable number of amino acids were observed between residues 292 and 304 of the

precursor protein in the group D prothrombin activators, plus another area of low homology in the heavy chain within residues 353 to 360 (figure 2.03).

In human factor Xa, 12 disulfide bonds have been identified in total, 7 in the light chain, 4 in the heavy chain and one inter-chain disulfide linkage (Davie *et al.*, 1991). All of these cysteine residues are conserved in all 7 snake sequences identified in this study, with the exception of a C216S substitution in the *H. stephensii* precursor protease, consistent with the previously published amino acid sequence of hopsarin D (P83370) (Rao *et al.*, 2003a). Similarly, vitamin K-dependent post-translational γ-carboxylation of glutamic acid (GLA) residues in the light chain has been described for human factor Xa and the group D prothrombin activators, including eleven GLA residues identified in the N-terminal domain of trocarin D (Joseph *et al.*, 1999). Results in figure 2.03 demonstrate that these eleven glutamic acid residues are also conserved in all Australian elapids with the exception of an E72K substitution in the *P. porphyriacus* precursor protein.

A further post-translational modification reported in the prothrombin activator of pseutarin C from *P. textilis* is an O-linked glycosylation of a serine at position 92 of the precursor protein that is also present in the other 6 elapid sequences (figure 2.03) (Joseph *et al.*, 2003). Similarly, an N-linked glycosylation of a heavy chain asparagine at position 254 of the pseutarin C precursor protein is also conserved amongst the other snakes with *P. porphyriacus* again being the exception. The three active site residues of human factor Xa are conserved within all snakes in this study, corresponding to positions H251, D309 and S406 of the *O. scutellatus* precursor. An activation peptide located between the light and heavy chain sequences in the precursor human factor X plays a key role in the folding and processing of the protease (Baugh and Krishnaswamy, 1996). A 27 amino acid sequence (approximately half that of the human equivalent), similarly positioned between the light and heavy chains, is also present and highly conserved in all snakes.

Comparative Analysis of Factor V-like Proteins

Based on the nucleotide sequence of the factor V-like non-enzymatic subunit of pseutarin C from *P. textilis*, amplification of similar full-length cDNA sequences for elapids involved in this study was attempted. An amplified product was only observed in *O. scutellatus*, *O. microlepidotus* and *P. textilis*, compatible with the fact that only group C prothrombin

71

activators contain their own factor V-like sequence (figure 2.05, lanes 1-3). Alignment of the deduced protein sequence from multiple cDNA clones demonstrated that there was 95.7% identity between the three snakes (figure 2.06).
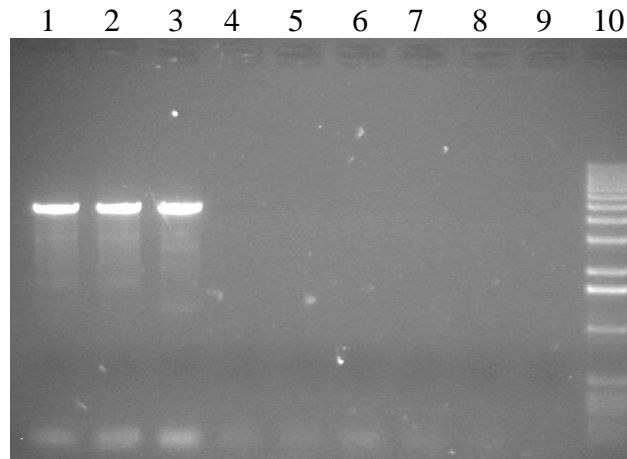
Figure 2.05. PCR amplification of a factor V-like transcript.
PCR amplification of the complete 4.4kb factor V-like transcript from venom gland cDNA isolated from 1) *P. textilis*, 2) *O. scutellatus*, 3) *O. microlepidotus*, 4) *N. scutatus*, 5) *T. carinatus*, 6) *P. australis*, 7) *P. porphyriacus,* 8) *H. stephensii*, 9) no template control and 10) molecular weight marker. Note the presence of a product only in the group C elapids.

The overall domain arrangement of the factor V-like protein observed in the *Oxyuranus* species was the same as that of mammalian coagulation factor V and the non-enzymatic subunit of pseutarin C (that is, A1-A2-B-A3-C1-C2) (Rao *et al.*, 2003b). The two thrombin cleavage sites reported in *P. textilis* (R772 and R817 in the precursor molecule) are again conserved between the three snakes. Rao *et al*. (2003) have recently demonstrated that the pseutarin C non-enzymatic subunit contains only a single conserved activated protein C (APC) cleavage site, whereas the mammalian equivalent has three sites required for the inactivation of factor Va (Kalafatis *et al.*, 1994). Both *P. textilis* and *O. microlepidotus* contain a lysine residue at this site whereas a conserved amino acid (arginine) is present in the *O. scutellatus* protein.

```
                         ┌──→  Signal peptide              ┌──→  A1 Domain.  Heavy Chain
O.scu. 1    MGRYSVSPVPKCLLLMFLGWSGLKYYQVNAAQLREYRLAAQLEDWDYNPQPEELSRLSESDLTFKKIVYREYELDFKQEKPRDELSGLLG  90
O.mic. 1    MGRYSVSPVPKCLLLMFLGWSGLKYYQVNAAQLREYRIAAQLEDWDYNPQPEELSRLSESELTFKKIVYREYELDFKQEKPRDELSGLLG  90
P.tex. 1    MGRYSVSPVPKCLLLMFLGWSGLKYYQVNAAQLREYHIAAQLEDWDYNPQPEELSRLSESDLTFKKIVYREYELDFKQEEPRDALSGLLG  90

O.scu. 91   PTLRGEVGDSLIIYFKNFATQPVSIHPQSAVYNKWSEGSSYSDGTSDVERLDDAVPPGQSFKYVWNITAEIGPKKADPPCLTYAYYSHVN  180
O.mic. 91   PTLRGEVGDTLIIYFKNFATQPVSIHPQSAVYNKWSEGSSYSDGTSDVERLDDAVPPGQSFKYVWNITAEIGPKKADPPCLTYAYYSHVN  180
P.tex. 91   PTLRGEVGDSLIIYFKNFATQPVSIHPQSAVYNKWSEGSSYSDGTSDVERLDDAVPPGQSFKYVWNITAEIGPKKADPPCLTYAYYSHVN  180

O.scu. 181  MVRDFNSGLIGALLICKEGSLNADGAQKFFNREYVLMFSVFDESKNWYRKPSLQYTINGFANGTLPDVQACAYDHISWHLIGMSSSPEIF  270
O.mic. 181  MVRDFNSGLIGALLICKEGSLNANGAQKFFNREYVLMFSVFDESKNWYRKPSLQYTINGFANGTLPDVQACAYDHISWHLIGMSSSPEIF  270
P.tex. 181  MVRDFNSGLIGALLICKEGSLNANGSQKFFNREYVLMFSVFDESKNWYRKPSLQYTINGFANGTLPDVQACAYDHISWHLIGMSSSPEIF  270

                                                                              │  A2 Domain
O.scu. 271  SVHFNGQTLEQNHYKVSTINLVGGASVTANMSVSRTGKWLISSLVAKHLQAGMYGYLNIKDCGNPDTLTRKLSFRERRIMKWEYFIAAE  360
O.mic. 271  SVHFNGQTLEQNHYKVSTINLVGGASVTANMSVSRTGKWLISSLVAKHLQAGMYGYLNIKDCGHPNTLTRKLSFRELRRIMNWEYFIAAE  360
P.tex. 271  SVHFNGQTLEQNHYKVSTINLVGGASVTADMSVSRTGKWLISSLVAKHLQAGMYGYLNIKDCGNPDTLTRKLSFRELMKIKNWEYFIAAE  360

O.scu. 361  EITWDYAPEIPSSVDRRYKAQYLDNFSNFIGKKYKKAVFRQYEDSNFTKPTYAIWPKERGILGPVIKAKVRDTVTIVFKNLASRPYSIYV  450
O.mic. 361  EITWDYAPEIPSSVDRRYKAQYLDNFSNFIGKKYKKAVFRQYEDGNFTKPTYAIWPKERGILGPVIKAKVRDTVTIVFKNLASRPYSIYV  450
P.tex. 361  EITWDYAPEIPSSVDRRYKAQYLDNFSNFIGKKYKKAVFRQYEDGNFTKPTYAIWPKERGILGPVIKAKVRDTVTIVFKNLASRPYSIYV  450

                                                          APC cleavage site        ↓
O.scu. 451  HGVSVSKDAEGAVYPSDPKENITHGKAVEPGQVYTYKWTVLDTDEPTVKDSECITKLYHSAVDMTRDIASGLIGPPLVCKRKALSIRGVQ  540
O.mic. 451  HGVSVSKDAEGAIYPSDPKENITHGKAVEPGQVYTYKWTVLDTDEPTVKDSECITKLYHSAVDMTRDIASGLIGPLLVCKLKALSVKGVQ  540
P.tex. 451  HGVSVSKDAEGAIYPSDPKENITHGKAVEPGQVYTYKWTVLDTDEPTVKDSECITKLYHSAVDMTRDIASGLIGPLLVCKHKALSVKGVQ  540

O.scu. 541  NKADVEQHAVFAVFDENKSWYLEDNIKKYCSNPSSVKKDDPKFYKSNVMYTLNGYASDRTEVWGFHQSEVVEWHLTSVGTVDEIVPVHLS  630
O.mic. 541  NKADVEQHAVFAVFDENKSWYLEDNIKKYCSNPSSVKKDDPKFYKSNVMYTLNGYASDRTEVLGFHQSEVVQWHLTSVGTVDEIVPVHLS  630
P.tex. 541  NKADVEQHAVFAVFDENKSWYLEDNIKKYCSNPSAVKKDDPKFYKSNVMYTLNGYASDRTEVLRFHQSEVVQWHLTSVGTVDEIVPVHLS  630

                                                 ┌──→  B Domain
O.scu. 631  GHTFLSKGKHQDILNLFPMSGESATVTMDNLGTWLLSSWGSCEMSNGMRLRFLDANYDDEDEGNEEEEEEDDGDIFADIFNPPEVVIKKEE  720
O.mic. 631  GHTFLSKGKHQDILNLFPMSGESATVTMDNLGTWLLSSWGSCEMSNGMRLRFLDANYDDEDEGNEEEEEEDDGDIFADIFSPPEVVKKKEE  720
P.tex. 631  GHTFLSKGKHQDILNLFPMSGESATVTMDNLGTWLLSSWGSCEMSNGMRLRFLDANYDDEDEGNEEEEEEDDGDIFADIFIPSEVVKKKEE  720

                          ↓  Thrombin cleavage site
O.scu. 721  VPVNFVPDPESDALAKELGLFDDEDNPK-QSRSEQTEDDEEQLMIASMLGLRSFKGSVAEEELKHTALALEEDAHASDPRIDSNSAHNSD  809
O.mic. 721  VPVNFVPDPESDALAKELGLLDDEDNPE-QSRSEQTEDDEEQLMIASVLGLRSFKGSVAEEELKHTALALEEDAHASDPRIDSNSARNSD  809
P.tex. 721  VPVNFVPDPESDALAKELGLIDDEGNPIIQPRREQTEDDEEQLMKASMLGLRSFKGSVAEEELKHTALALEEDAHASDPRIDSNSARNPD  810

                 ┌──→  A3 Domain (and thrombin cleavage site).  Light Chain
O.scu. 810  DIAGRYLRTINRRNKRRYYIAAEEVLWDYSPIGKSQVRSLPAKTTFKKAIFRSYLDDTFQTPSTGGEYEKHLGILGPIIRAEVDDVIEVQ  899
O.mic. 810  DIAGRYLRTINRRNKRRYYIAAEEVLWDYSPIGKSQVRSLPAKTTFKKAIFRSYLDDTFQTPSTGGEYEKHLGILGPIIRAEVDDVIEVQ  899
P.tex. 811  DIAGRYLRTINRGNKRRYYIAAEEVLWDYSPIGKSQVRSRAAKTTFKKAIFRSYLDDTFQTPSTGGEYEKHLGILGPIIRAEVDDVIETQ  900

O.scu. 900  FRNLASRPYSLHAHGLLYEKSSEGRSYDDNSPELFKKDDAIMPNGTYTYVWQVPPRSGPTDNTEKCKSWAYYSGVNPEKDIHSGLIGPIL  989
O.mic. 900  FRNLASRPYSLHAHGLLYEKSSEGRSYDDNSPELFKKDDAIMPNGTYTYVWQVPPRSGPTDNTEKCKSWAYYSGVNPEKDIHSGLIGPIL  989
P.tex. 901  FKNLASRPYSLHAHGLLYEKSSEGRSYDDKSPELFKKDDAIMPNGTYTYVWQVPPRSGPTDNTEKCKSWAYYSGVNPEKDIHSGLIGPIL  990

O.scu. 990  ICQKGMIDKYNRTIDIREFVLFFMVFDEEKSWYFPKSDKSTCEEKLIGVQS-RHTFPAINGIPYQLQGLMMYKDENVHWHLLNMGGPKDV  1078
O.mic. 990  ICQKGMIDKYNRTIDIREFVLFFMVFDEEKSWYFPKSDKSTCEEKLIGVQSSHHTFPAINGIPYQLQGLMMYKDENVHWHLLNMGGPKDI  1079
P.tex. 991  ICQKGMIDKYNRTIDIREFVLFFMVFDEEKSWYFPKSDKSTCEEKLIGVQS-LHTFPAINGIPYQLQGLTMYKDENVHWHLLNMGGPKDI  1079

                                                          │  C1 Domain
O.scu. 1079 HVVNFHGQTFTEEGREDNQLGVLPLLPGTFASIKMKPSKIGTWLLETEVGENQERGMQALFTVIDKDCKLPMGLASGIIQDSQISASGHV  1168
O.mic. 1080 HVVNFHGQTFTEEGREDNQLGVLPLLPGTFASIKMKPSKIGTWLLETEVGENQERGMQALFTVIDKDCKLPMGLASGIIQDSQISASGHV  1169
P.tex. 1080 HVVNFHGQTFTEEGREDNQLGVLPLLPGTFASIKMKPSKIGTWLLETEVGENQERGMQALFTVIDKDCKLPMGLASGIIQDSQISASGHV  1169

O.scu. 1169 GYWEPKLARLNNTGMFNAWSIIKKEHEHPWIQIDLQRQVVITGIQTQGTVQLLKHSYTVEYFVTYSKDGQNWITFKGRHSKTQMHFEGNS  1258
O.mic. 1170 EYWEPKLARLNNTGMFNAWSIIKKEHEHPWIQIDLQRQVVITGIQTQGTVQLLKHSYTVEYFVTYSKDGQNWITFKGRHSETQMHFEGNS  1259
P.tex. 1170 GYWEPKLARLNNTGKYNAWSIIKKEHEHPWIQIDLQRQVVITGIQTQGTVQLLQHSYTVEYFVTYSEDGQNWITFKGRHSETQMHFEGNS  1259

                                          │  C2 Domain              -- Ca²⁺ binding
O.scu. 1259 DGTTVKENHIDPPIIARYIRLHPTKFYNTPTFRIELLGCEVEGCSVPLGMESGAIKDSEITASSYKKTWWSSWEPFLARLNLKGRTNAWQ  1348
O.mic. 1260 DGTTVKENHIDPPIIARYIRLHPTKFYNTPTFRIELLGCEVEGCSVPLGMESGAIKNSEITASSYKKTWWSSWEPFLARLNLEGGTNAWQ  1349
P.tex. 1260 DGTTVKENHIDPPIIARYIRLHPTKFYNRPTFRIELLGCEVEGCSVPLGMESGAIKNSEITASSYKKTWWSSWEPSLARLNLEGGTNAWQ  1349

O.scu. 1349 PKVNNKDQWLQIDLQHLTKITSIITQGATSMTTSMYVKTFSIHYTDDNSTWKPYLDVRTSMEKVFTGNINSDGHVKHFFNPPILSRFIRI  1438
O.mic. 1350 PEVNNKDQWLQIDLQHLTKITSIITQGATSMTTAMYVKTFSIHYTDDNSTWKPYLDVRTSMEKVFTGNINSDGHVKHFFKPPILSRFIRI  1439
P.tex. 1350 PEVNNKDQWLQIDLQHLTKITSIITQGATSMTTSMYVKTFSIHYTDDNSTWKPYLDVRTSMEKVFTGNINSDGHVKHFFKPPILSRFIRI  1439

O.scu. 1439 IPKTWNQYIALRIELFGCEVF  1459
O.mic. 1440 IPKTWNQYIALRIELFGCEVF  1460
P.tex. 1440 IPKTWNQYIALRIELFGCEVF  1460
```

Figure 2.06. Alignment of Factor V-like proteins.

Comparison of the factor V-like non-enzymatic subunit of the prothrombinase complex identified in the group C containing Australian. Arrows mark the boundaries of the different domains and conserved cysteines forming putative disulfide linkages are shaded gray.

The association of factors Xa and Va on a phospholipid bilayer, forming the prothrombinase complex, is a key step in the blood coagulation process (Steen, 2002). Kalafatis and Beck (2002) identified a binding site for factor Xa within the heavy chain of human factor Va between amino acid residues 323 and 331. The corresponding residues in the elapid sequences (amino acids E354→I362 in the precursor molecule) are completely conserved with one another, and the first 8 of these residues identical to the corresponding human sequence, with the final amino acid being a hydrophobic residue in both instances (V→I). Another key component of the prothrombinase complex are phospholipids, which have been predicted from 3-dimensional structural analysis to bind to the surface exposed loop KKSWW in mammalian coagulation factor Va. Mutagenesis of these residues identified both tryptophans, which are absolutely conserved in all three elapid sequences, as important in interactions with phospholipids (Nicolaes *et al.*, 2000). Of the 7 disulfide linkages reported for human factor Va, six are conserved in alignments with the group C snake sequences (figure 2.06).

mRNA Transcription of Factor X- and Factor V-like Components from Australian Elapids

As outlined above the Australian elapid prothrombin activators under investigation fall into two categories, group C which are complete prothrombinase complexes and group D that require phospholipids, calcium ions and mammalian coagulation factor Va for activity (Chester and Crawford, 1982). The results in figure 2.07 demonstrate that all 3 group C snakes express the factor V-like subunit mRNA. It is of interest that the highest level of expression of factor V-like protein is observed in *P. textilis* (approximately 6 times greater than that of the *Oxyuranus* species). This reflects the proportion of prothrombinase activator in these species' venom (Masci *et al.*, 1988). As expected, it was not possible to detect expression of this factor at the mRNA level in any of the four snakes with group D prothrombin activators.

Transcription of the factor X-like protease was detected in both group C and group D snakes with some degree of variability (figure 2.07). The venom of *P. australis* has been shown to be largely anticoagulant in clinical pathology and laboratory studies (Dambisya *et al.*, 1995; Tan and Ponnudurai, 1990). Thus it was not surprising that mRNA for neither factor X or factor V-like proteins were expressed in the venom gland of this snake. It is also evident that neither of these factors are expressed in the liver of *O. scutellatus*.
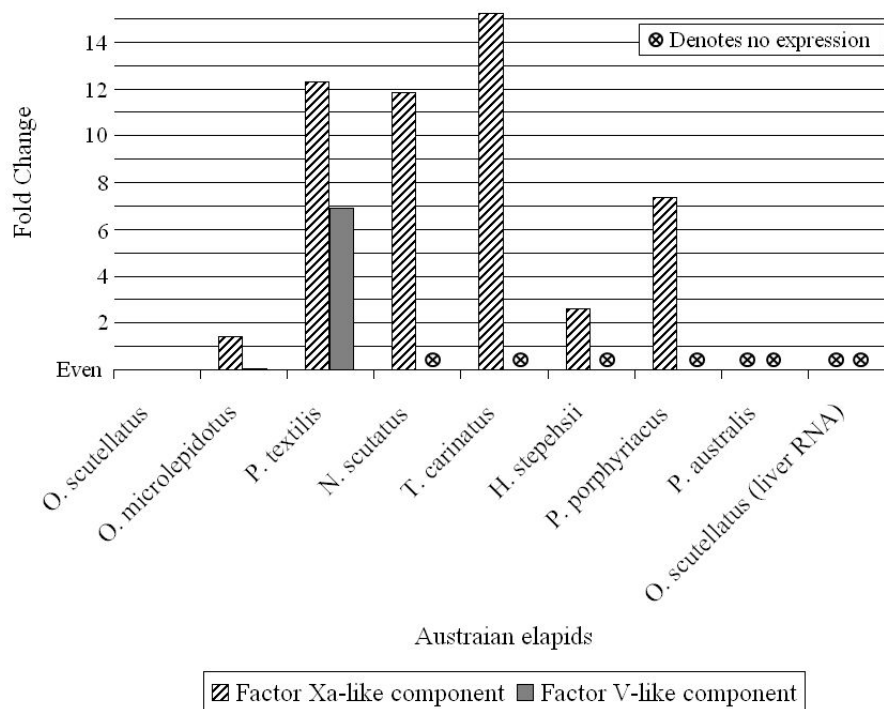
Figure 2.07. mRNA expression levels of components of the prothrombinase complex.
Expression levels were determined by quantitative PCR analysis in Australian elapid snakes
relative to that of the coastal taipan (*O. scutellatus*). A liver RNA sample from *O. scutellatus*
is included as a control.

Relative Expression of Factor Xa-like Proteases in Australian Snake Venoms

To further investigate the prothrombin activator complex in Australian elapids, the expression
of the factor Xa catalytic and factor Va-like non-catalytic components was examined in the
venom of these snakes. Initially 10μg of crude venom was run under reducing conditions of a
12% SDS-polyacrylamide gel and examined via staining with colloidal coomassie (figure
2.08). It is evident from the gel that the venom of all Australian elapids contain a multitude of
proteins of varying molecular mass. Other notable features include the similar banding
patterns between the taipan species (*O. scutellatus* and *O. microlepidotus*, lanes 2 and 3) and
the significant proportion of factor Xa-like protease in the venom of *P. textilis* as can be
determined by comparison to the purified form in lane 10.

The presence of the components of the factor X-like prothrombin activator was further
investigated by immunoblotting with a range of specific antibodies. Under non-reducing
conditions, the total mass of the factor Xa-like protease (light plus heavy chain linked by a

single disulfide bond) in the venom of group C elapids (*O. scutellatus*, *O. microlepidotus* and *P. textilis*) corresponded in size to the purified *P. textilis* protein, and was somewhat greater than that of the group D proteases (figure 2.09A, lanes 1-3 versus 4-7). This antibody (which had been raised against a recombinant form of the heavy chain from *P. textilis*) failed to detect protein in *P. australis* as expected (lane 8). To gain an accurate estimate of molecular size, the venom was run under reducing conditions and protein detected with the same antibody (figure 2.09B). The heavy chain of the factor Xa-like protease (Xa-HC) was approximately the same size in all three group C snakes (lanes 1-3) and again corresponded in size to the purified *P. textilis* protein (lane 9). The molecular weight of this protein was lower in the other group D elapids (lanes 4-7), consistent with deletions observed in the heavy chain as described in figure 2.03. The greater molecular weight change evident in the heavy chain of *P. porphyriacus* under reducing conditions (lane 7) does not appear to be solely due to changes in the number of amino acids, but might be explained by a reduced degree of post-translational modifications or a different pattern of cleavage.
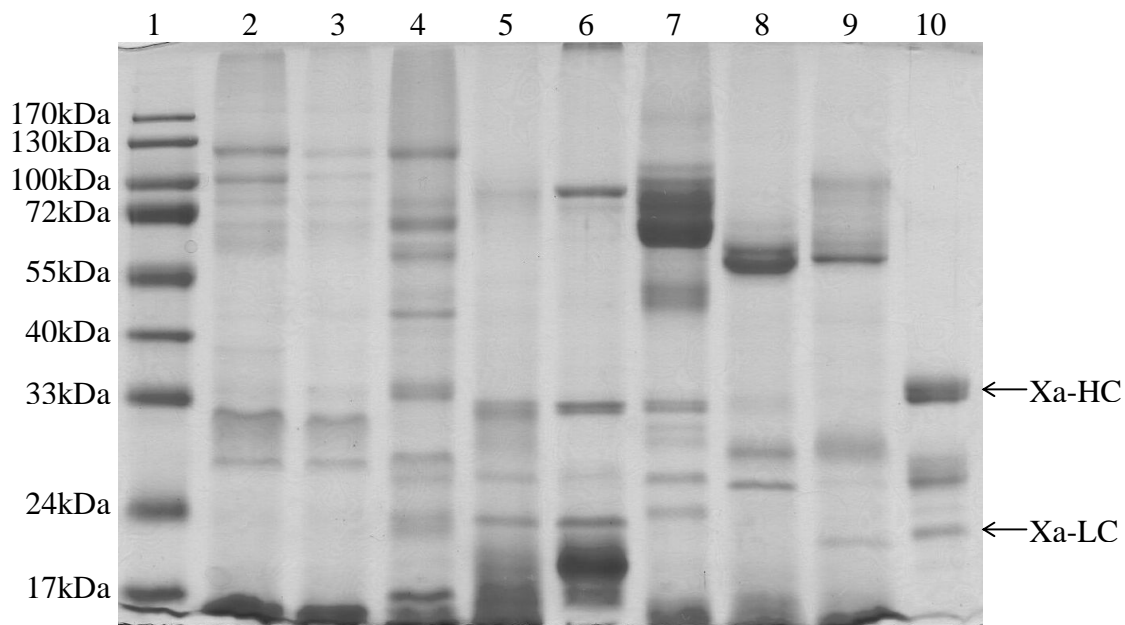
Figure 2.08. Australian elapid snake whole venom.
Venoms were run on a 12% SDS-PAGE under reducing conditions and stained with colloidal coomassie. Lanes were loaded as follows: 1) Molecular weight marker, 2) *O. scutellatus*, 3) *O. microlepidotus*, 4) *P. textilis*, 5) *N. scutatus*, 6) *T. carinatus*, 7) *H. stephensii*, 8) *P. porphyriacus*, 9) *P. australis* and 10) purified *P. textilis* factor Xa-like protease. HC = Heavy chain; LC = Light Chain.
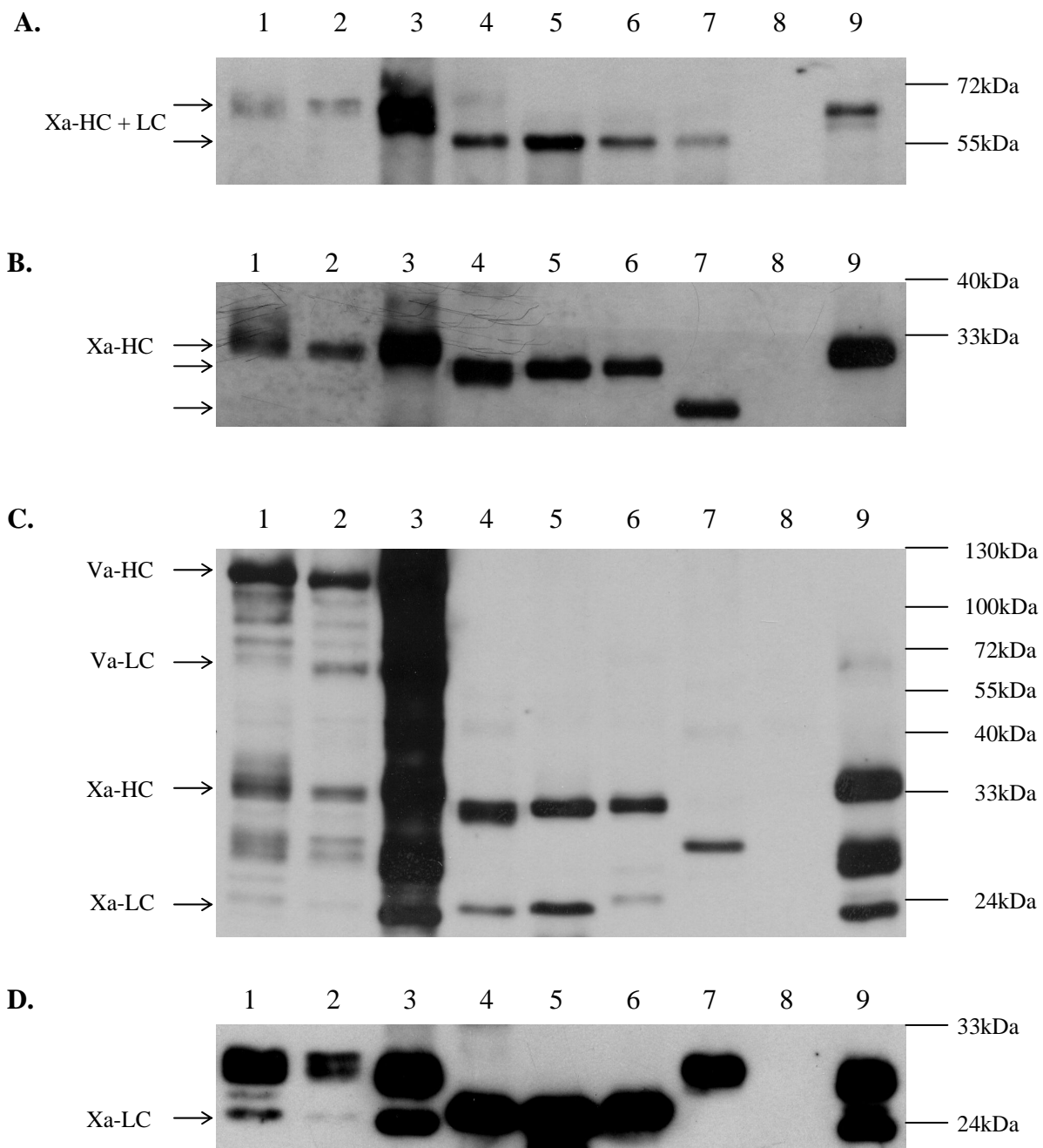
Figure 2.09. Immunoblot detection of the Australian snake prothrombin activators.
In all immunoblots, each lane was loaded with 10μg of venom protein in the following order:
1) *O. scutellatus*, 2) *O. microlepidotus*, 3) *P. textilis*, 4) *N. scutatus*, 5) *T. carinatus*, 6) *H. stephensii*, 7) *P. porphyriacus*, 8) *P. australis* and 9) purified *P. textilis* factor Xa-like protease. **(A)** Detection with an anti-heavy chain factor X-like protease antibody run under non-reducing gel conditions. **(B)** Detection with an anti-heavy chain antibody under reducing conditions. **(C)** Detection with anti-sera raised against the complete snake prothrombin activator from *P. textilis* under reducing conditions. **(D)** Detection of proteins with a commercial antibody raised against GLA residues under reducing conditions.

Amongst the group C prothrombin activators, *P. textilis* had the highest level of expression of the factor Xa-like protease, being approximately 5-fold greater than either *O. scutellatus* or *O. microlepidotus* per µg of total venom (figure 2.09B, compare lanes 3 with 1 and 2). This approximates the expression observed at the mRNA level (figure 2.07). The amount of the factor Xa-like protease was variable in all group D snakes, with the greatest amount of protein being observed in *T. carinatus*, again agreeing with observations at the level of the mRNA.

The results in figure 2.09C demonstrate the presence of the light chain in both groups of snakes, however no signal was detectable in *P. porphyriacus* using an antibody raised against the total prothrombin activator complex of *P. textilis*. This band was verified as being the light chain by immunoblotting with an antibody specific for γ-carboxylated sites which are known to be present close to the N-terminus of the light chain (figure 2.09D) (Persson *et al.*, 1993). The antibody used in figure 2.09C also detected proteins of approximately 65kDa and 100kDa which correspond in size to the light and heavy chains of the non-enzymatic factor V-like subunit, and were only present in the group C venoms as expected.

Expression of Recombinant Factor X-like Protease from *O. scutellatus*

Expression of the full-length construct for the factor X-like protease from *O. scutellatus* was performed to investigate the processing of this molecule and determine its activity. The full-length *O. scutellatus* protease cDNA (ProP-HC-AP-LC), designated Pre-Prot, was cloned into the pMIB vector for transfection and recombinant expression in SF9 insect cells. This construct contains a honeybee melittin secretion signal for efficient export into the growth media as well as a V5 epitope and his-tag on the C-terminus for detection and purification respectively. A second construct was also prepared without the snake propeptide sequence (Mat-Prot) (figure 2.10). Stable cell lines were established by selection with blastocidine. Recombinant protein secreted into the supernatant by the transfected SF9 cells was recovered and partially purified by nickel column chromatography (figure 2.11). The presence of the his-tag purified recombinant protease construct used in subsequent immunoblots and RVV activation studies is evident in lane 13.

```
                     Honeybee Melittin Secretion Signal                                           ->Propeptide
  1  ATGAAATTCTTAGTCAACGTTGCCCTTGTTTTTATGGTCGTATACATTTCTTACATCTATGCCGGCATGCTAAGCTTGATGGCTCCTCAA
     M  K  F  L  V  N  V  A  L  V  F  M  V  V  Y  I  S  Y  I  Y  A  G  M  L  S  L  M  A  P  Q

 91  CTACTCCTCTGTCTGATCCTCACTTTTCTGTGGAGTCTCCCAGAGGCCGAAAGTAATGTATTCTTAAAAAGCAAAGTGGCAAATAGATTT
     L  L  L  C  L  I  L  T  F  L  W  S  L  P  E  A  E  S  N  V  F  L  K  S  K  V  A  N  R  F

                             -> Light chain
181  TTGCAAAGAACAAAACGAGCTAATTCACTGTATGAGGAATTTAGATCTGGAAACATTGAAAGGGAATGCATTGAGGAGAGATGTTCAAAA
     L  Q  R  T  K  R  A  N  S  L  Y  E  E  F  R  S  G  N  I  E  R  E  C  I  E  E  R  C  S  K

271  GAAGAAGCCAGGGAGGTATTTGAAGATGACGAGAAAACTGAGACCTTCTGGAATGTTTATGTAGATGGGGATCAGTGTTCATCAAACCCC
     E  E  A  R  E  V  F  E  D  D  E  K  T  E  T  F  W  N  V  Y  V  D  G  D  Q  C  S  S  N  P

361  TGTCATTATCGCGGGACATGCAAAGATGGCATTGGTAGCTATACCTGTACCTGCTTGTCTGGCTATGAAGGGAAAAACTGTGAACGTGTC
     C  H  Y  R  G  T  C  K  D  G  I  G  S  Y  T  C  T  C  L  S  G  Y  E  G  K  N  C  E  R  V

451  TTATATAAGTCCTGCAGAGTGGACAATGGTAACTGTTGGCACTTCTGCAAACCTGTTCAAAACGATATTCAGTGTTCATGTGCTGAAGGT
     L  Y  K  S  C  R  V  D  N  G  N  C  W  H  F  C  K  P  V  Q  N  D  I  Q  C  S  C  A  E  G

                                                                                    ->  AP
                                                                           CCGGACATCAGGATAGTA
                                                                           P  D  I  R  I  V
541  TACCTTTTGGGAGAGGATGGGCACTCTTGTGTTGCTGGAGGTAACTTTTCATGTGGTAGAAATATCAAAACAGGAACAAGAGGGAAGCA
     Y  L  L  G  E  D  G  H  S  C  V  A  G  G  N  F  S  C  G  R  N  I  K  T  R  N  K  R  E  A

                                                                       -> Heavy chain
631  AGTCTGCCTGACTTTGTGCAGTCCCAGAATGCAATTTTGCTGAAAAAATCTGATAATCCAAGCCCTGATATCAGAATTGTTAATGGAATG
     S  L  P  D  F  V  Q  S  Q  N  A  I  L  L  K  K  S  D  N  P  S  P  D  I  R  I  V  N  G  M

721  GACTGCAAACTGGGTGAATGTCCGTGGCAGGCAGTTCTGGTAGATGAAAAGGAAGATGCGTTTTGTGGAGGAACAATTTTGAGTCCCATC
     D  C  K  L  G  E  C  P  W  Q  A  V  L  V  D  E  K  E  D  A  F  C  G  G  T  I  L  S  P  I

811  TATGTGCTTACTGCAGCCCACTGCATTAACCAGACCAAGATGATTTCAGTTGTTGTAGGGGAAATAAACATATCAAGAAAAAACCCCGGA
     Y  V  L  T  A  A  H  C  I  N  Q  T  K  M  I  S  V  V  V  G  E  I  N  I  S  R  K  N  P  G

901  CGTCTTCTTTCTGTGGATAAAATATATGTGCATCAAAAATTTGTTCCTCCCAAAAAAGGCTATGAATTCTATGAAAGTTTGATCTTGTC
     R  L  L  S  V  D  K  I  Y  V  H  Q  K  F  V  P  P  K  K  G  Y  E  F  Y  E  K  F  D  L  V

991  AGCTATGACTATGATATAGCCATCCTCCAAATGAAGACCCCTATCCAGTTCTCTGAAAATGTGGTTCCTGCCTGCCTTCCCACAGCTGAT
     S  Y  D  Y  D  I  A  I  L  Q  M  K  T  P  I  Q  F  S  E  N  V  V  P  A  C  L  P  T  A  D

1081 TTTGCCAACCAAGTCCTCATGAAACAAGATTTTGGCATCGTTAGTGGATTTGGGCGTATTTTCGAAAAAGGACCTCAATCTAAAACACTT
     F  A  N  Q  V  L  M  K  Q  D  F  G  I  V  S  G  F  G  R  I  F  E  K  G  P  Q  S  K  T  L

1171 AAAGTCCTTAAGGTTCCTTATGTGGACAGGCACACCTGCATGCTTTCCAGCGAATCTCCAATTACTCCAACTATGTTCTGTGCTGGCTAT
     K  V  L  K  V  P  Y  V  D  R  H  T  C  M  L  S  S  E  S  P  I  T  P  T  M  F  C  A  G  Y

1261 GATACTCTGCCTCGAGATGCATGCCAGGGAGACAGTGGGGGGCCCCACATCACTGCATACAGAGATACCCACTTTATTACTGGGATTGTC
     D  T  L  P  R  D  A  C  Q  G  D  S  G  G  P  H  I  T  A  Y  R  D  T  H  F  I  T  G  I  V

1351 AGCTGGGGGGAAGGATGTGCACAGACAGGCAAATATGGTGTTTACACAAAAGTGTCCAAATTCATCCTTTGGATAAAAAGAATAATGCGT
     S  W  G  E  G  C  A  Q  T  G  K  Y  G  V  Y  T  K  V  S  K  F  I  L  W  I  K  R  I  M  R

                                                <-                  -> V5 Epitope
1441 CAAAAGCTACCCAGTACAGAGTCAAGCACTGGTCGGCTCTCTAGAGGGCCCTTCGAAGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTC
     Q  K  L  P  S  T  E  S  S  T  G  R  L  S  R  G  P  F  E  G  K  P  I  P  N  P  L  L  G  L

            <-                ->6xHis Tag  <-
1531 GATTCTACGCGTACCGGTCATCATCACCATCACCATTGA
     D  S  T  R  T  G  H  H  H  H  H  H  *
```

Figure 2.10. Recombinant factor X-like oscutarin C protease construct.

The construct was based on the protease cloned from *O. scutellatus* into the pMIB expression vector. Three constructs were produced: one full length protease (Pre-Prot), one without the underlined propeptide (Mat-Prot) and one full length clone with the cleavage site surrounding the light chain and activation peptide mutagenised to mimic that of the cleavage site between the activation peptide and heavy chain (Pre-Mut). AP = Activation Peptide.
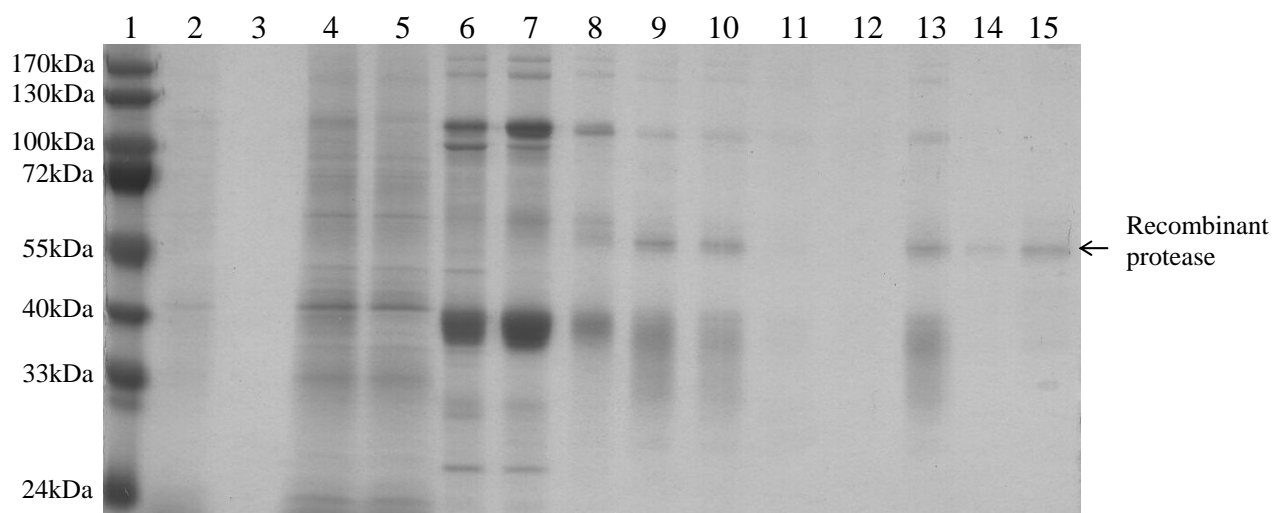
Figure 2.11. Nickel purification of the recombinant protease construct Pre-Prot.
Fractions were run on a 12% SDS-PAGE under non-reducing conditions and stained with colloidal coomassie, loading as follows: 1) Molecular weight marker, 2) SF9 media supernatant, 3) Concentration filtrate (no protein), 4) Pre-nickel column load, 5) Post-nickel column run-off, 6-8) Washes 1 to 3 of the column, 9-12) Elutions 1 to 4 from the column, 13) Concentrated recombinant protease, 14) 1µg BSA control and 15) 5µg BSA control.

Recombinant protein secreted into the media was detected by immunoblotting with a commercial antibody specific for the V5 epitope attached to the C-terminus of the heavy chain (figure 2.12A). A single band was observed under non-reducing conditions for both constructs (with and without the propeptide) indicating that both the honeybee melittin secretion signal and the snake propeptide were cleaved off upon export out of the insect cell. A single band was also observed with the V5-antibody under reducing conditions for both constructs. The size of this protein band was approximately 35kDa, which corresponds to a fusion of the heavy chain with the V5 epitope and his-tag. The difference in migration under reducing and non-reducing conditions is compatible with the expected processing of the precursor molecule (HC-AP-LC) where the activation peptide is cut and the light and heavy chains are complexed by a single disulfide linkage (Rudolph *et al.*, 1997). Russell's Viper venom (RVV) has previously been employed to remove the activation peptide in the conversion of mammalian factor X to its mature form (Messier *et al.*, 1991; Kisiel *et al.*, 1976). Treatment of the products obtained from both constructs with RVV led to an increased migration of the recombinant protein in both cases under non-reducing conditions (figure 2.12A, compare lanes 1 and 3 with lanes 2 and 4). This is constistent with the activation

80

peptide being still attached to either the light or heavy chain. Since treatment with RVV failed to alter the size of the protease under reducing conditions where only the heavy chain is detected, it is unlikely that the activation peptide is still attached to the heavy chain. This was confirmed by immunoblotting purified recombinant protease with anti-sera against native *P. textilis* prothrombin activator (figure 2.12B). Treatment with RVV increased the migration of the light chain to a position the same as that observed for the light chain of purified *P. textilis* protease. This shift in migration can be accounted for by the removal of a fragment the size of the activation peptide (less than 3kDa). As expected, RVV did not change the size of the heavy chain. The reduced migration of the recombinant heavy chain compared to the corresponding native heavy chain (figure 2.12B, compare lane 1 with 2 and 3) is due to the presence of the C-terminal V5 epitope and his-tag.

Given the above evidence that the activation peptide was still attached to the light chain in both recombinant constructs, but was efficiently cleaved from the heavy chain by the insect cells, this cleavage site was mutagenised by substituting the light chain/activation peptide sequence with that of the cleavage site between the activation peptide and heavy chain (figure 2.10). Stable cell lines expressing this protease (Pre-Mut) were established, but the protein produced by this construct was not processed and migrated at a position on the gel corresponding to an uncleaved combination of the light chain, activation peptide and heavy chain (figure 2.12C).

The N-terminal region of the light chain of mammalian coagulation factor Xa contain eleven glutamic acid residues that are γ-carboxylated, with conservation of these sites within oscutarin C, the prothrombin activator identified in *O. scutellatus* venom. There is evidence that these post-translational modifications are important to the function of the mature protease (Brown *et al.*, 2002). The results of the immunoblot in figure 2.12D, performed with an antibody specific for GLA residues, demonstrate that the light chain of the purified native protease is γ-carboxylated. The higher molecular weight fragment appears to correspond to a degradation product of the precursor molecule and is also detected with anti-sera against the native protease (figure 2.12B). This is supported by the recognition of a single product under non-reducing conditions (figure 2.12D). There was no evidence for γ-carboxylation of the recombinant proteases, due to an absence of the specific carboxylase enzyme within SF9 insect cells (Roth *et al.*, 1993).
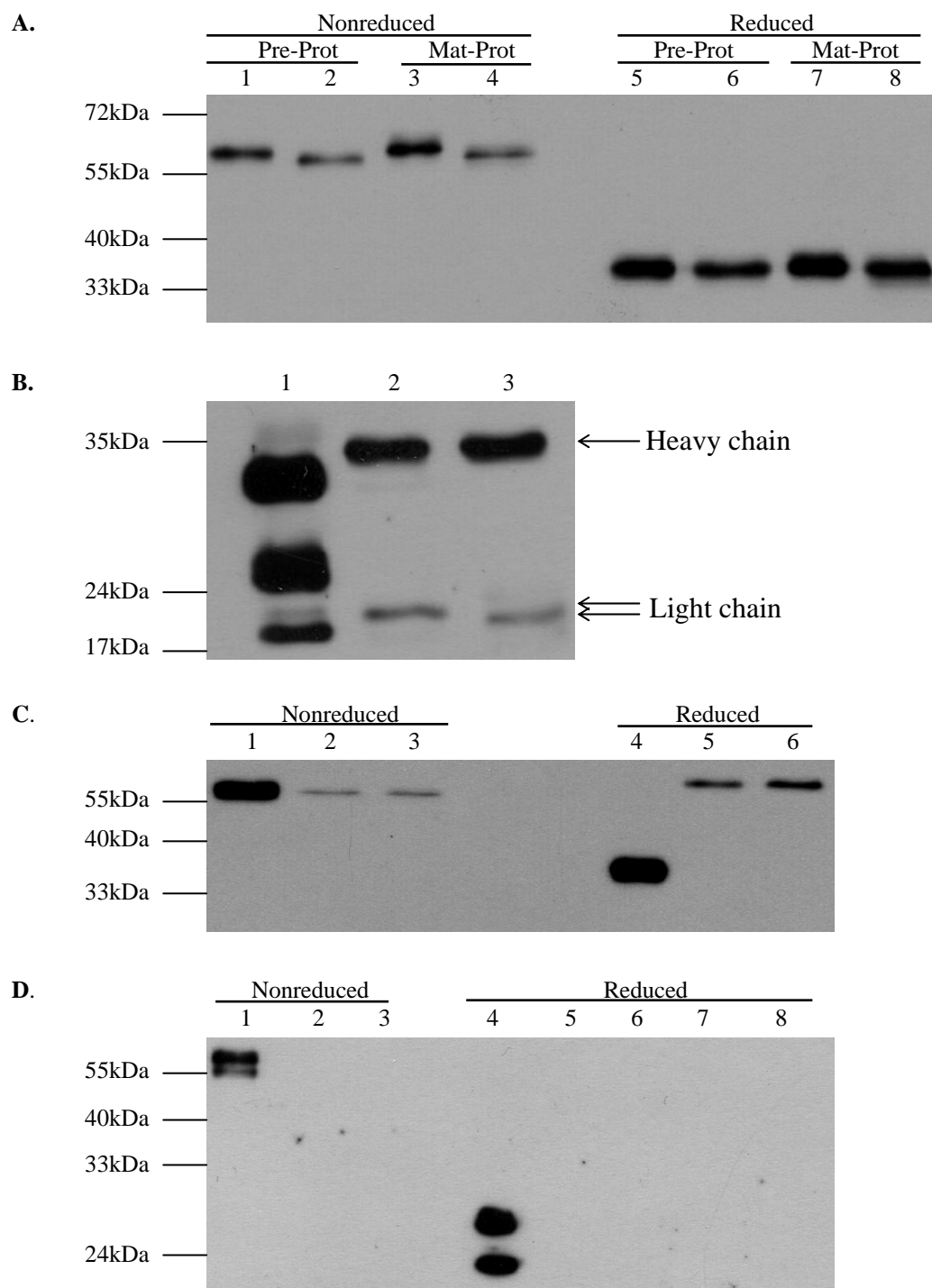
Figure 2.12. Immunoblot detection of the recombinant factor Xa-like protease.
 (A) Anti-V5 antibody was used to detect the V5 tag present on the heavy chain of two
constructs, with and without propeptide (Pre-Prot and Mat-Prot respectively), where the
protein in lanes 2, 4, 6 and 8 have been activated with RVV. Note the shift in size upon
cleavage of the activation peptide (AP) in the reduced form, which detects both light and
heavy chains, but not in the non-reduced form which detects only the heavy chain. **(B)** Use of

anti-sera raised against native factor Xa, under reducing gel conditions, detecting 1) native *P. textilis* factor Xa-like protease, 2) inactive recombinant protease and 3) recombinant protease activated with RVV. Note the shift in the size of the light chain in the recombinant protease upon activation with RVV. **(C)** Detection of the mutagenic protease (Pre-Mut, lanes 2, 3, 5 and 6) compared to that of the original Pre-Prot construct (lanes 1 and 4) with the anti-V5 antibody. Note that reduction of Pre-Mut does not result in separation of the light and heavy chains, indicating that neither of the cleavage sites surrounding the AP are processed. **(D)** Detection of the protease with anti-γ-carboxyglutamic acid antibody which detects the native *P. textilis* protease light chain but not the purified recombinant oscutarin C. Purified *P. textilis* protease (lanes 1 and 4), recombinant protease with (lanes 3 and 6) and without (lanes 2 and 5) RVV activation and recombinant protease present in insect cell media supernatant with (lane 8) and without (lane 7) RVV activation.

Activity of Recombinant Factor X-like Protease

Activity of the recombinant protease was determined by the ability to hydrolyse the chromogenic substrate S-2765. No activity was observed with either of the original *O. scutellatus* factor X-like recombinant proteases (Pre-Prot and Mat-Prot). However when these recombinant proteases were pre-incubated with RVV, comparable activity was observed with the S-2765 substrate for both constructs (table 2.02). The rate of cleavage however, was only somewhere between 1.4% and 1.7% of that of the native protease isolated from *P. textilis* venom. In the protease construct containing the mutagenised cleavage site, no activity was observed with or without treatment with RVV. Thromboelastograph studies performed with all three recombinant protease constructs prior to activation with RVV revealed they did not enhance the rate of blood coagulation, consistent with the inactivity observed in the chromogenic assay. It was not possible to test activity of the recombinant proteins after RVV activation with the thromboelastograph as RVV is a potent stimulator of blood coagulation itself and hence would interfere with subsequent results.

Protease Construct	S2765 Activity (U/min/µg of protein)
Pre-Prot - no treatment	0.0007
- RVV activated	0.0628
Mat-Prot - no treatment	0.0000
- RVV activated	0.0725
Pre-Mut - no treatment	0.0005
- RVV activated	0.0005
RVV only	No activity
SF9 supernatant only	No activity
P. textilis native protease	4.4000

Table 2.02. S-2765 activity rates for recombinant protease constructs.
Activity of the recombinant protein in the S-2765 chromogenic assay, measured in absorbance units/min/µg of protein. The Pre-Prot and Mat-Prot constructs had an activity of 1.4% and 1.6%, respectively, of that on the purified native *P. textilis* protease.

Discussion

The mammalian prothrombinase complex, composed of factor Xa in association with its cofactors calcium, phospholipids and factor Va, plays a central role in the process of blood coagulation (Davie *et al.*, 1991; Tracy *et al.*, 1985). An activity related to that of the prothrombinase complex has been well documented in the venom of the Australian elapid snakes (Braud *et al.*, 2000; Matsui *et al.*, 2000). The activity of this prothrombin activator complex has been characterised functionally in both the group C proteases, which require calcium and phospholipids to clot mammalian blood and the group D prothrombin activators, which have an additional requirement for factor Va for activity (Kini *et al.*, 2001b). This study describes a comparative molecular analysis of the factor X- and factor V-like components of the prothrombin activators from both subgroups of elapids. The results demonstrate that there is a high degree of sequence relatedness in the factor X-like proteases in all seven snake species across the two groups. This is evident throughout the entire length of the precursor molecule, with the exception of two short regions within the heavy chain that differentiate the proteases from each group (figure 2.03, amino acids 292→304 and 353→360 of the precursor protein). The deduced amino acid sequence of the proteases described here display 78% to 96% identity to the precursor catalytic subunit of pseutarin C from *P. textilis*, recently reported by Rao *et al.* (2004). In that study, cDNA sequence encoded a precursor

protein of 449 amino acids compared to the 467 amino acid protein for *P. textilis* in this study (Filippovich *et al.*, 2005). This difference in size can be attributed to the presence of a termination codon (TAA) in the Rao *et al*. (2004) sequence compared to a lysine (AAA) at position 450 of all clones of the precursor *P. textilis* sequence (figure 2.03). Given that this lysine is conserved across all seven species of snake studied here, it is likely the stop codon described by Rao *et al*. (2004) is due to an error in sequencing or an aberrant clone.

The amino acid sequence for the factor Xa-like catalytic subunit from *T. carinatus* (trocarin D) and *H. stephensii* (hopsarin D) determined by protein sequencing has also been reported (Rao *et al.*, 2003a; Joseph *et al.*, 1999). Comparison with the deduced amino acid sequences from multiple cDNA clones identified in the present study reveal that the propeptide and activation peptide sequences are absent from these protein sequences, consistent with their cleavage post-translationally during the formation of an active molecule. The propeptide in both snakes was 40 amino acids in length and highly conserved, as was the activation peptide (27 residues). The deduced amino acid sequence for *T. carinatus* and *H. stephensii* described in figure 2.03 contain an 11 residue segment of the heavy chain (S260→S270) not identified in either the trocarin D or hopsarin D protein sequences. Given that the cDNA data described here demonstrates the presence of this sequence in all seven snakes, it would indicate that this fragment is an inherent part of the mature heavy chain. Except for this deletion, the published protein sequence for trocarin D is 100% identical to the transcript identified in this study (Joseph *et al.*, 1999). Only three amino acid differences were observed when comparisons were made between hopsarin D and its cDNA counterpart (Rao *et al.*, 2003a). In both of these sequences (actual and deduced protein) a serine is present at position 216 of the precursor instead of a cysteine conserved in all other snakes. This cysteine is also conserved in mammalian coagulation factor Xa and is involved in a heavy chain intra-disulfide linkage (MacGillivray and Fung, 1989).

Mammalian factor X is produced in an inactive precursor form containing a propeptide. In order to generate an active molecule (factor Xa), cleavage of the propeptide is required initially, followed by removal of an activation peptide sequence between the light and heavy chains, giving rise to a correctly folded mature protease (Chattopadhyay *et al.*, 1992). Since the corresponding precursor protein identified in snake venom contains a similar domain arrangement, it is likely that these molecules are processed in a similar fashion. The purification and identification by amino acid sequencing of the heavy and light chains of the

factor Xa-like protease from *T. carinatus* supports a similar mechanism of processing (Joseph *et al.*, 1999). They identified the N-terminal sequence of the light chain as SNS which is consistent with cleavage on the C-terminal side of an arginine residue (R↓SNS), deduced from results described by Rao *et al.* (2004) and the data described in this report using cDNA cloning of the factor X-like protease. It is of interest that this cleavage site (R↓ANS) is identical between human factor X and the group C prothrombin activator sequences described here, whereas a single amino acid difference is present in the group D proteases (R↓SNS). The cleavage site surrounding the activation peptide, which was observed to be highly conserved in all seven snake species, are also closely related to the corresponding sites in the human factor X protease. In addition the H-D-S catalytic triad, the 11 analogous sites known to be γ-carboxylated in the mammalian factor X protease and the cysteine residues involved in disulfide linkages are also highly conserved in all species, supporting the importance of these amino acids/modifications in the function of the protein.

Mammalian factor Va enhances the prothrombin converting activity of the group D proteases but is not required by the group C activators as they contain their own factor V-like protein. The group C prothrombin activators have previously been isolated from *O. scutellatus* and *P. textilis* and are high molecular weight complexes made up of multiple subunits (Masci *et al.*, 1988). The non-enzymatic subunit of the group C prothrombin activators enhances the catalytic activity of the snake factor Xa-like protease in the conversion of prothrombin to thrombin. Rao and Kini (2002) purified the non-enzymatic subunit of pseutarin C from *P. textilis* and revealed that it had a domain arrangement similar to that of mammalian coagulation Va with a homology of approximately 55%. This study has described cloning of the factor V-like cDNA from two other members of the group C snakes (*O. scutellatus* and *O. microlepidotus*) and demonstrated that it had 96% identity with the *P. textilis* protein coding sequence also cloned as part of this study. The *P. textilis* clone was observed to be 100% homologous to that reported by Rao *et al.* (2003). It was not possible to identify a similar transcript by PCR in any of the group D venoms, or in *P. australis*, given that a factor V-like protein has never been detected in the venom of these snakes. The overall domain arrangement (A1-A2-B-A3-C1-C2) is conserved between all group C complexes identified in this study and is similar to that observed in the mammalian protein. A 30 amino acid signal peptide is 100% identical between the three snakes, which is of considerable interest since this high degree of conservation is also observed within the propeptide for the factor X-like

component of the complex in the same snakes. While the signal peptide bears little relatedness to the corresponding human factor V sequence, the cleavage site between this peptide and the heavy chain (A↓AQLR) is conserved between human and snake species. The cleavage sites at either end of the B domain however, whilst conserved amongst the snakes, display little similarity to the human homolog. Furthermore, the B domain is conserved in size between the snakes (126 to 127 amino acids) but is significantly smaller than that of the human precursor protein (882 amino acids).

Previous data suggest that the prothrombin activator represents as much as 40% of the total venom protein from *P. textilis*, which is several-fold greater than that observed for the corresponding complex in *O. scutellatus* (Masci *et al.*, 1988). This difference in the amount of prothrombin activator was confirmed in the present study at both the mRNA and protein levels for *O. scutellatus*, and in addition for another member of the same genus, *O. microlepidotus*. This was the case for both the factor X- and factor V-like proteins, which were present in approximately equivalent proportions in both *Oxyuranus* species.

As expected the presence of the non-catalytic factor V-like protein was not detected in any of the group D snake venoms, however, the heavy chain of the factor X-like protease was present in all four snakes. It is obvious that the molecular size of the heavy chain under reducing conditions is less than that observed in the group C protein (figures 2.09B and 2.09C). This is particularly evident in the protein from *P. porphyriacus*, which migrates at an apparent molecular weight of 29kDa. In the case of *N. scutatus*, *T. carinatus* and *H. stephensii* this molecular weight difference can be explained by a short deletion in the heavy chain gene sequence and the introduction of a premature stop codon 33 nucleotides shorter than the open reading frame for the group C transcripts (figure 2.03). In the case of the *P. porphyriacus* gene, a 39 nucleotide deletion in the heavy chain coding sequence could account for at least some of the molecular size difference. An additional contribution to the size change may be due to a N254T substitution within the precursor protein, which would be predicted to result in the loss of an N-linked glycosylation site in this species. The apparent discrepancy between the migration of the factor Xa-like protein under reducing and non-reducing conditions in *P. porphyriacus* relative to the other group D snakes (compare figure 2.09A to 2.09B) is difficult to explain but may be due to differences in post-translational modifications affecting running conditions or an alternate pattern of cleavage. Although the light chain for the factor Xa-like protease is evident in all of the other 6 snakes using anti-sera

raised against the total prothrombin activator from *P. textilis*, this band was not detected in *P. porphyriacus*. However, a γ-carboxylated band was identified at a position of higher molecular weight than that expected of the light chain, and which appeared to correspond in size to one similar to that of the heavy chain. Given that this modification is specific to the light chain, and since 10 of the 11 γ-carboxylation sites are conserved in the light chain of *P. porphyriacus*, it suggests the pattern of cleavage during processing of the factor X-like protein in this elapid differs to that of the others, even though this is not evident in the primary amino acid sequence. Neither component of the prothrombin activator was detected in the venom of *P. australis* , confirming the inability to detect mRNA in this species and hence the absence of procoagulant activity in this snake.

Messier *et al*. (1991) have previously described the expression of a recombinant form of mammalian factor X but could only produce an active product in a chromogenic assay after prior treatment with Russell's Viper venom (RVV). This finding was confirmed by Rudolph *et al*. (1997) who expressed human factor X in human kidney cells. As observed in the present study, *O. scutellatus* recombinant factor X expressed in insect cells also revealed a requirement for RVV for activation of its protease activity. Under these conditions the activity of the recombinant protease was only approximately 1.7% of the purified native *P. textilis* protease in the same assay. Attempts to alter the cleavage between the light chain and activation peptide via mutagenesis resulted in a failure to observe any activity, even after treatment with RVV. This may suggest a specific interaction between these two sites to allow cleavage to occur. These results, together with the failure to observe γ-carboxylation of the light chain within the recombinant protease, suggest that expression in insect cells will not provide a viable approach for the expression of active protease.

The family *Elapidae* contains almost 300 individual species of which approximately 91 are found in Australia (Shea, 1999; Keogh *et al.*, 1998). Indeed almost all known venomous Australian snakes are members of the *Elapidae* family and are amongst the most toxic in the world (Broad *et al.*, 1979). There are 3 major toxic components characterised from Australian elapid venoms, including prothrombin activating enzymes, phospholipases and potent peptidic neurotoxins (Fry, 1999). The prothrombin activators are responsible solely for the procoagulant activities of these venoms, which contain no thrombin-like enzymes and prior classification of these venoms into group C and D has been on the basis of cofactor

requirement alone (Chester and Crawford, 1982). Clearly it is desirable to have an additional means for the classification of these species in order to understand their divergence and evolutionary development. The cloning and sequence analysis of genes implicated in the coagulation process described here represents a useful approach for molecular classification. Phylogenetic analysis of the primary factor X-like protease sequence from a select representation of Australian elapids confirms their previous classification on the basis of their cofactor requirement (figure 2.04). Using the sequence analysis approach described here, the elapids with group C proteases cluster with a significantly high degree of identity (91%) and differ from the group D proteases with a number of subtle variations including alternate cleavage recognition sites between the propeptide and light chain as well as other significant changes within the heavy chain. When bootstrap analysis was applied, the relationship *within* the group C sequences is less well supported with an apparent paraphyly of the *Oxyuranus* species. However it should be noted that the bootstrap values in establishing this relationship are low, and direct sequence comparison supports a closer relationship between the two taipans than that suggested by the phylogenetic tree (table 2.01). Using immunoblotting alone it is possible to distinguish the two groups on the basis of molecular size of the factor Xa heavy chain. Interestingly the overall domain arrangement of the protease is retained amongst both groups of snakes and indeed with that of the human sequence. Conservation of this structure between mammals and a non-hepatic, non-mammalian source (that is, venom) indicates its importance for the production of functionally active molecules in the coagulation cascade. This also holds true of the active site residues and other sections of the protein implicated in substrate/cofactor binding and post-translational modification.

The functional differences observed between the venom of the group C and group D elapids can be attributed to the absence of a factor V-like cofactor in the group D prothrombin activators, as further confirmed by this study. Sequence results of this protein show a high degree of identity within the group C snakes as well as a significant level of conservation with its mammalian homolog, again confirming the importance of this structure in molecular interactions with activated factor X. In short, this project describes a new means of classification of the group C and group D prothrombin activators from elapids snakes using different molecular markers including DNA sequence analysis, presence or absence of the factor V-like protein and the molecular size of the factor Xa-like heavy chain.

In conclusion this study has detailed the cloning and characterisation of both the factor X- and factor V-like cDNA components of the prothrombin activator from Australian group C and group D elapids. This data reveals a high degree of preservation in domain arrangement with the corresponding mammalian proteins, which is in keeping with their conserved function. The molecular classifications described here provide additional important information on the evolutionary relationship of these subclasses of elapid snake.

CHAPTER 3

Identification and Analysis of Venom Gland Specific Genes Within the Coastal Taipan (*Oxyuranus scutellatus*) and Related Species

Australian terrestrial snakes, which belong almost exclusively to the elapid family, contain amongst the most potently toxic and highly evolved venoms known. The well described clinical effects of Australian snake envenomation include neurological symptoms such as disorientation, flaccid paralysis and respiratory failure in association with multiple coagulopathic effects, most notably massive disseminated intravascular coagulation in a number of species (Fry, 1999). Secondary clinical features resulting from elapid bites include haemotoxicity, myotoxicity, nephrotoxicity and neurotoxicity contributing to the nausea, pain and swelling experienced by snake bite victims (Ponraj and Gopalakrishnakone, 1995; Francis *et al.*, 1993). However despite these well characterised clinical features within mammalian systems, little is known about individual components of Australian elapid venoms, particularly at the molecular level.

In addition to the factor Xa-like prothrombin activators described in chapter 2, elapid venoms are known to house a multitude of other peptidic toxins, the combined effect of which contribute to immobilise, kill, and partially digest the prey. Examples of these molecules include families of neurotoxins and phospholipase A_2 enzymes, L-amino acid oxidases as well as the recently identified venom natriuretic peptides (see chapter 1 for a review of each) (Fry *et al.*, 2005; Tsetlin, 1999; Harris, 1997; Stiles *et al.*, 1991). The study of snake venom toxins has previously focussed on a number of different objectives, foremostly neutralising the adverse toxic effects of the venom in a bitten subject. It is only recently that venoms have become a mainstream source of unique pharmacological molecules that may be of diagnostic and/or therapeutic benefit (Kini, 2002). Toxins are useful in the drug discovery process as they arise from a non-mammalian source, yet often specifically target functional mammalian systems in a highly potent, specific and stable manner.

Given the diversity of proteins and polypeptides known to exist within the venoms of Australian snakes, and given the relative potency of these components in altering mammalian homeostasis and as yet their relatively unstudied nature, this study aimed to identify and further characterise at a molecular level those components within the venom of the coastal taipan (*O. scutellatus*) and related Australian elapids. To this end a cDNA microarray was

established from the coastal taipan venom gland mRNA for the purposes of cross species comparisons of gene expression levels and the identification of novel compounds/toxins which may be of diagnostic or therapeutic benefit. Results from this study will provide a systematic comparison of those components within the venom gland of Australian snakes and should also shed light on the evolutionary relationship between species. A comparative analysis of the microarray data may aid in the identification of novel compounds that may be of pharmacological potential and provide information for the detection and treatment of snake bite.

Methods and Materials

cDNA Library Construction and Amplification

The SMART™ cDNA Library Construction Kit (Clontech, Palo Alto, California) was used for the production of full-length cDNA libraries from two snakes including the common brown (*P. textilis*) and coastal taipan (*O. scutellatus*) from venom gland total RNA. The library from *O. scutellatus* was ultimately selected for use in the creation of the cDNA microarray chip, with details of the production and screening of the array provided below.

First Strand Synthesis and Amplification by LD PCR

First strand cDNA was synthesised using 1μg of venom gland total RNA (isolated as previously described) with 1μM SMART II™ oligonucleotide (5'-AAG CAG TGG TAT CAA CGC AGA GTG GCC ATT ACG CCG GGG-3') and 1μM CDS III/3' primer (5'-ATT CTA GAG GCC GAG GCG GCC GAC ATG-d(T)$_{30}$ VN -3'). Primers were annealed at 72°C for 2min, followed by a quick chill on ice. cDNA was then synthesised with the incorporation of the primer sites in 50mM Tris-HCl pH 8.3, 3mM $MgCl_2$, 75mM KCl, 2mM dithiothreitol (DTT), 1mM of a dNTP mix and 200 units of Superscript II in a final reaction volume of 10μL, incubating at 42°C for 60min. The cDNA was then amplified via long-distance PCR (LD PCR). A 2μL aliquot of first strand cDNA was used as template in a reaction with 2μL of Advantage 2 Polymerase mix in 40mM Tricine-KOH pH 8.7, 15mM KOAc, 3.5mM $Mg(OAc)_2$, 375ng BSA, 0.005% Tween, 0.005% Nonidet P-40 and 0.2mM

dNTP mix, amplifying with 200nM 5'PCR primer (5'-AAG CAG TGG TAT CAA CGC AGA GT-3') and 200nM CDS III/3' primer in a final reaction volume of 100μL. The PCR mix was thermocycled at 95°C followed by 32 cycles of 95°C for 5sec and 68°C for 6min. The sample was then analysed by running 5μL of amplified cDNA on a 1% TAE agarose gel stained with ethidium bromide.

Proteinase K and Sfi *1 digestion*

To inactivate residual DNA polymerase activity a total of 50μL of amplified cDNA (approximately 3μg) was added to 40μg of Proteinase K and digested at 45°C for 20min. The mixture was then taken up to a final volume of 100μL with sterile water and the amplified cDNA purified by phenol:chloroform (1:1 v/v) precipitation. Briefly, 100μL of phenol was added to the sample, mixed by gentle inversion for 1min and centrifuged at 16,100g for 5min to separate the phases. The uppermost aqueous phase was then recovered and the amplified cDNA was again purified with phenol:chloroform under centrifugation. The sample was precipitated from the aqueous phase by the addition of 10μL 3M sodium-acetate, 26μg of glycogen and 100μL of 95% ethanol followed by centrifugation at 16,100g for 20min at room temperature. Supernatant was removed and the pellet further washed with 80% ethanol, and after drying briefly was resuspended in 12.5μL of sterile water.

The amplified cDNA was then digested with *Sfi* 1 restriction enzyme (New England Biolabs, Beverly, Massachusetts) to create overlapping ends for future cloning into *Sfi* 1-digested λTripleEx2™ vector. 12.5μL of purified, amplified cDNA was digested with 150 units of *Sfi* 1 in 10mM Tris-HCl pH 7.9, 50mM NaCl, 10mM $MgCl_2$, 1mM DTT and 25ng of BSA in a total volume of 25μL. The digestion mixture was incubated at 50°C for 2hours 45min.

Size Fractionation

Size fractionation of the amplified cDNA sample (which was necessary to remove transcripts smaller than 600bp in size so as to obtain a high quality library of full-length clones) was then performed via a two-step process. Initially the amplified cDNA sample was run on a 1.5% TAE agarose gel stained with ethidium bromide and the smear above 600bp in size, as determined by a comparable 1kb molecular weight marker, was excised into four pieces and

purified with a QIAEX II Gel Extraction kit as previously described. The final elution volume of 80μL was ethanol precipitated to remove contaminants and eluted in 20μL of sterile water. A 2μL aliquot of this sample was again examined on a 1.5% TAE agarose gel before proceeding.

The remaining 18μL of sample was subjected to further size fractionation with a CHROMA SPIN™-400 Column (Clontech, Palo Alto, California). CHROMA SPIN columns allow for the purification of higher molecular weight DNA based on the principle of gel filtration chromatography. The column was prepared for drip procedure as described in the user manual, then washed with 700μL of column buffer (10mM Tris-HCl pH 8, 1mM EDTA). The cDNA sample was loaded, followed by the addition of a further 100μL of column buffer. After the removal of short transcripts and contaminants under gravity, 600μL of column buffer was added and a total of fifteen 40μL fractions were collected. 3μL of each fraction was analysed on a 1.25% TAE agarose gel, and fractions 6 to 9, which contained the cDNA, pooled together. The pooled fractions were then ethanol precipitated with 14μL 3M sodium-acetate pH 5.4, 26μg of glycogen and 400μL of 95% ethanol in dry ice for 80min, centrifuged at 16,100g for 20min and eluted in 7μL of sterile water after drying briefly.

Ligation into λTripleEx2™Vector

The λTripleEx2™ vector contains asymmetrical *Sfi* 1sites that eliminate adapter ligation and facilitate directional cloning of the size fraction cDNA, in addition to blue/white screening of recombinants. A total of 3μL of size fractioned, digested cDNA was incubated with 500ng of λTripleEx2 ARMS, 200 units of T4 DNA Ligase, buffered to a final concentration with 50mM Tris-HCl pH 7.8, 10mM $MgCl_2$, 10mM DTT and 250ng BSA in a total reaction volume of 5μL. The ligation mixture was incubated at 16°C overnight.

Packaging of λ DNA into Phage and Transfection into XL1 Blue E. coli

The total ligation mixture from above was then packaged into a bacteriophage system for the production of a gene library using the Packagene® Lambda DNA Packaging System from Promega (Madison, Wisconsin). The process involved the combination of the total 5μL of

ligation mixture with 50μL of phage Package Extract, followed by incubation at 22°C for

3hours. 410μL of Phage buffer (20mM Tris-HCl pH 7.4, 100mM NaCl and 10mM MgSO$_4$)

and 25μL of chloroform were then added. Serial ten-fold dilutions of the cDNA library were

then prepared from 1:100 up to 1:100,000 for transfection into *E. coli*, and the remaining

packaging mixture stored with 17.5μL DMSO and 2.5μL gelatin. A 1mL starter culture of

XL1 Blue *E. coli* (with tetracycline resistance) was prepared the previous day and grown

shaking at 37°C in LB broth with 0.2% Maltose and 10mM MgSO$_4$. This culture was used to

inoculate 50mL of pre-warmed LB plus 0.2% Maltose and 10mM MgSO$_4$ and grown for a

further 3hours at 37°C. A 100μL aliquot of these cells was then transfected with 100μL of

diluted packaging mixture and recovered at 37°C for 30min. The transfection mixture was

added to 4mL of fresh molten TB Top agar (add 2g Trypticase peptone, 1g NaCl, 1.6g Agar

to 200mL water, autoclave, then add 2mL of 1M MgSO$_4$) in the presence of 20mg/ml IPTG

and 100mM X-Gal for colour selection. The top agar was then poured onto LB Agar plates,

set and incubated at 37°C overnight, where upon they were examined for blue and white

plaques the following morning.

PCR Screening of the SMART cDNA Library

After selection of the appropriate titre of the packaged amplified cDNA Library to ensure an

even spread of individual white plaques, the efficiency of ligation and size and redundancy of

the library was examined by phage PCR and sequencing. Selected plaques were cored from

the agar plates and incubated in 300μL of phage buffer at 37°C shaking for 3hours. A 3μL

aliquot of this preparation was then used in a PCR with 1unit of AmpliTaq gold buffered in

10mM Tris-HCl pH 8.3, 50mM KCl, 2mM MgCl$_2$, 200μM dNTPs and 7.5ρmol of each of the

5'-LD forward and 3'-LD reverse primer. All reactions were run at a final volume of 25μL

with appropriate no-template controls. The PCR was thermocycled 95°C for 10min, followed

by 30 cycles of 95°C for 20sec, 57°C for 20sec and 72°C for 3min, with a final extension of

72°C for 7min. PCR products were then examined on a 1.25% TAE agarose gel stained with

ethidium bromide to examine the number of recombinant inserts in each plaque core and their

average size. PCR products were then purified with a QIAquick PCR Purification kit

(Qiagen, Hilden, Germany) as described below. Purified product could then be sequenced as

previously described with either the 5'-LD forward primer or 3'-LD reverse primer for a

sample selection of clones from the amplified cDNA library to examine the potential sequence redundancy of transcripts.

Library Amplification

Upon preliminary screens of both the *P. textilis* and *O. scutellatus* cDNA libraries, the *O. scutellatus* library was selected for further amplification and isolation of clones for the purposes of producing a cDNA microarray. This was due to the average increased insert size and variability for this library. An XL1 Blue starter culture in LB/ MgSO$_4$ (200μL) was transfected with 100μL of a 1:600 dilution of the λphage cDNA library and recovered at 37°C for 30min. The mixture was added to either 4mL or 8mL of TB Top agar with IPTG and X-Gal and poured onto either 900mm or 1500mm LB agar plates respectively, allowed to set and incubated at 37°C overnight. Plaques containing clones with inserts, as indicated by their white colour, were then cored the following morning into 300μL of phage buffer in a 96-well plate format and shaken at 37°C for 3hours. Prior to storage of the master block at -70°C, 21μL of DMSO was added to each well and mixed by pipetting up and down. In this fashion, a total of 4,800 plaques were isolated and stored at -70°C in a total of 50 blocks.

PCR product was then amplified from each isolated clone, with the incorporation of a 5' amino C6 group on the primer so as to allow fixation of the PCR product to the glass slide when arraying the chip. A 100μL PCR reaction was prepared with 12μL isolated phage in buffer as template. The reaction mixture contained 1μL of recombinant taq (see below for purification protocol) seeded with 0.1 unit of AmpliTaq, buffered with 10mM Tris pH8.8, 50mM KCl, 0.1% Triton X-100, 2.5mM MgCl2, 200nM dNTPs and 0.1ρmol of each of the Sticky 5'LTD forward primer (5'-C$_6$-CTC GGG AAG CGC GCC ATT GTG TTG GT-3') and Sticky 3'LTD reverse primer (5'- C$_6$-ACG ACT CAC TAT AGG GCG AAT TGG CC-3'). Again, PCR reactions were performed in a 96-well plate format on all 4,800 clones. Cycling conditions were as follows: 95°C for 5min, then 35 cycles of 95°C for 20sec, 67°C for 20sec, 72°C for 3min with a final extension of 72°C for 7min. 5μL of PCR product was then loaded on a 1.25% TAE agarose gel stained with ethidium bromide to confirm amplification of the insert.

All PCR plates were isopropanol precipitated to remove unincorporated primers and dNTPs and to concentrate PCR products according to the following protocol. PCR plates were placed open in a 50°C oven overnight to reduce the volume from 100μL to approximately 20μL. 100μL of isopropanol was added to each well and PCR product precipitated at -70°C overnight, then the plates centrifuged at 3,100g for 40min at 4°C. Isopropanol was decanted and the pellet air-dried followed by resuspending in a solution containing 4x sodium chloride-sodium citrate (SSC) and 0.1% sarcosyl. Plates were then stored at -20°C awaiting microarray production.

PCR Purification

Purification of PCR products prior to sequencing without the necessity of gel extraction or cloning was performed with a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Briefly, the PCR sample was mixed with 5 volumes of buffer PB, placed in a QIAquick column and centrifuged at 16,100g for 1min. The column was then washed with 750μL of buffer PE, dried and the sample eluted with 35μL of elution buffer (10mM Tris-HCl pH 8.5) by further centrifugation whereupon it was stored at -20°C.

Preparation of Taq DNA Polymerase

An overnight culture of pRecTaq cloned into an inducible expression vector and transformed into XL1 blue *E. coli* cell stock was grown in 50mL of Terrific broth (10g trypticase peptone, 5g yeast, 2.31g KH_2PO_4 and 12.54g K_2HPO_4 in a final volume of 1L of water) shaking at 37°C with ampicillin to a final concentration of 100 μg/mL and tetracycline to a final concentration of 7.5μg/mL. This culture was then used to inoculate 250mL of Terrific broth and incubated shaking at 37°C until the absorbance reading at 600nm reached on optical density of 0.3. Taq polymerase expression was then induced with 5mM isopropyl beta-D-thiogalactopyranoside (IPTG) and incubated further at 37°C until the O.D. at 600nm reached 0.5 to 1. The culture was then centrifuged at 2,900g in a Beckman Coulter JA10 rotor to pellet the cells, which were then frozen of dry ice for 10min. The cell pellet was resuspended in TE buffer pH 8 (10mM Tris-HCl and 1mM ethylene diamine tretraacetic acid (EDTA)) to a final concentration of 160 O.D. units/mL. Cells were then sonicated on ice at 30 second intervals, then diluted in an equal volume of detergent solution comprised of 10mM Tris pH

8, 50mM KCl, 0.5% NP40, 0.5% Tween 20 and 1mM EDTA. This mixture was heated at 75°C for 35min, chilled on ice for 15min then centrifuged at 16,100g for 10min at 4°C. Supernatant containing the purified recombinant taq polymerase was then collected and resuspended in an equal volume of 100% glycerol before storage at -20°C for subsequent use in PCR.

Microarray Chip Production

The amplified cDNA library from *O. scutellatus* containing 4,800 PCR products in 96-well plate format was then spotted onto 42 slides to form the microarray chips to be used in the cross species comparisons of RNA expression. The initial stages of microarray chip production involved the preparation of the glass microscope slides, which were cleaned with a mixture of 2.5M sodium hydroxide (NaOH) and 57% ethanol, mixing for 2hours, followed by six rinses with fresh sterile water to remove all traces of the cleaning solution. The slides were then coated in poly-L-lysine solution (140mL poly-L-lysine, 140mL phosphate buffered saline (PBS) and 1120mL water) for 2hours followed by a brief rinse in water. The slides were then centrifuged for 7min at 600rpm to remove excess moisture and dried for 10min at 45°C. They were then stored for 7 days before the polylysine coat was examined under the scanner before use.

The 4,800 PCR clones were then arrayed onto the chip in duplicate using an automated inhouse GMS 417 arrayer (Genetic Microsystems Inc.), with the remaining PCR product being stored at -20°C again for future runs. The DNA was UV cross-linked to the glass slides with a Biorad crosslinker set to 65mJ. The slides were then immersed in a blocking solution composed of 3.6g succinic anhydride, 250mL 1-methyl-2-pyrrolidinone (Sigma-Aldrich, St Louis, Missouri) and 6mL 1M sodium borate pH 8, on an orbital shaker rotating at 50rpm for 20min. The slides were then transferred to 95°C water for 2min proceeded by a brief rinse in ice cold 95% ethanol. Again the slides were dried by centrifugation at 600rpm for 7min, whereupon they were stored in a cool dark box for a minimum of a week prior to use in RNA hybridisations.

<u>RNA Amplification</u>

First and Second Strand cDNA Synthesis

Venom gland RNA samples to be hybridised to the microarray chip were first amplified in a linear fashion to produce the large quantities of sample that the experimental procedure required. Selected RNA samples included those isolated from *P. textilis*, *O. scutellatus*, *O. microlepidotus*, *P. australis*, *P. porphyriacus*, *N. scutatus* and *T. carinatus* as previously described, in addition to RNA samples which had also been isolated from the livers of *O. scutellatus* and *O. microlepidotus*. Approximately 1μg of RNA, as determined by spectrophotomeric analysis of absorbance at 260nm, was incubated with 1μg of PAGE purified T7amp primer (5'-AAA CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG CGC $T_{(15)}$-3') (Sigma-Aldrich, St Louis, Missouri) in a total volume of 11μL at 65°C for 10min. The sample was cooled on ice before incubation with 200 units of Superscript III RNase H$^-$ Reverse Transcriptase buffered in 50mM Tris-HCl pH 8.3, 72.5mM KCl, 3mM $MgCl_2$ and 500nM dNTPs. The final reaction volume of 20μL was thermocycled at 37°C for 5min, 45°C for 5min then 10 cycles alternating between 60°C for 2min and 55°C for 2min.

Prior to second strand synthesis, a master mix was prepared for each reaction, composed of 40 units of *E. coli* DNA Polymerase 1, 2 units of RNase H in second strand buffer (at a final concentration of 20mM Tris-HCl pH 6.9, 4.6mM $MgCl_2$, 90mM KCl, 150nM β-NAD$^+$, 10mM $(NH_4)_2SO_4$) and 200μM dNTPs made up to final volume of 130μL with DEPC-water (Invitrogen, Mt Waverly, Australia). This master mix was added to the 20μL first strand reaction from above and incubated at 16°C for 2hours. The reaction was then stopped with 7.5μL of 1M HaOH, 2mM EDTA heated to 65°C for 10min.

Sample Extraction and Precipitation

The double stranded cDNA transcript was then purified via phenol:chloroform:isoamyl (25:24:1) precipitation with the use of phase-lock gel tubes to ensure the isolation of sample upon centrifugation. Briefly, a total of 150μL of organics was added to the second strand reaction and mixed via pipetting, which was then centrifuged at 16,100g for 5min. DNA was

precipitated from the aqueous phase upon its removal by the addition of 70μL of 7.5M ammonium acetate in DEPC-water plus 1mL absolute ethanol at -20°C. The sample was pelleted by centrifugation at 16,100g for 20min and washed further with 500μL absolute ethanol with a 5min spin, briefly air dried and resuspended in 8μL DEPC-water.

In Vitro *Transcription*

In vitro transcription off the second strand template to form large quantities of unlabelled amplified RNA (aRNA) was performed with the T7 MEGAscript™ kit from Ambion (Austin, Texas) according to manufacturer's instructions. A 12μL master mix containing 2μL of T7 enzyme mix, 8μL of a 18.75mM dNTP mix (note with UTP and not TTP) and 2μL of 10x reaction buffer was prepared and mixed with the double-stranded cDNA produced above, incubating at 37°C overnight. The following morning, DNA was digested from the sample via the addition of 50 units of DNAse 1 (Invitrogen, Mt Waverly, Australia), incubating for a further 15min at 37°C. The reaction was stoped via the addition of 115μL DEPC-water and 15μL ammonium acetate stop solution (5M ammonium acetate, 100mM EDTA). The RNA was purified via the addition of 150μL phenol:chloroform:isoamyl (25:24:1), centrifuging at 16,100g for 5min in a phase-lock gel. The RNA containing aqueous phase was then precipitated by addition of 150μL isopropanol, mixing well and incubating for a minimum of 30min at -20°C. Following centrifugation at 16,100g for 15min at 4°C, the pellet was washed with -20°C 80% ethanol, repelleted at 4°C and air dried briefly before being resuspended in 13μL DEPC-water. Quality of the aRNA was then investigated on a 1.25% TAE agarose gel stained with ethidium bromide and the yield quantitated by examining the absorbance at 260nm of a 1:100 dilution on a spectrophotometer. Samples were stored at -70°C awaiting analysis via hybridisation to the *O. scutellatus* microarray chip.

Microarray Hybridisations and Analysis

RNA hybridisation to the chip was performed using the indirect labelling method for microarrays, whereby Cy3 monofunctional dye-labelled sample RNA was hybridised to the chip in conjunction with Cy5 dye-labelled *O. scutellatus* venom gland aRNA as a reference RNA source in duplicate runs. Samples analysed on the microarray chip include aRNA isolated from the venom glands of *O. microlepidotus*, *P. textilis*, *P. porphyriacus*, *P. australis*,

N. scutatus and *T. carinatus*. Amplified RNA isolated from the livers of *O. scutellatus* and *O. microlepidotus* was also hybridised to the chip to detect genes whose expression levels were increased in the venom gland as well as venom gland total RNA from *O. scutellatus* as a reference to ensure no inherent bias in the RNA amplification process.

cDNA Synthesis and Incorporation of aa-dUTP

For each microarray hybridisation, a total of 2.5μg of RNA (as determined by spectrophotomeric analysis at 260nm) was incubated at 70°C for 10min with 2.5μg of Oligo-dT primer in a final volume of 18.5μL, then quick chilled on ice. cDNA was then synthesised with the incorporation of amino allyl labelled dUTP (aa-dUTP) in a 4:1 ratio with dTTP (Sigma-Aldrich, St Louis, Missouri). A 50x aa-dUTP/dNTP stock was previously prepared by resuspending aa-dUTP in 0.1M KPO_4 and adding to dNTPs at a final concentration of 23.25mM. The primed RNA sample was incubated for 2min at 42°C after the addition of 0.6μL of aa-dUTP/dNTP stock buffered in first strand buffer (50mM Tris-HCl pH 8.3, 72.5mM KCl and 3mM $MgCl_2$) and in the presence of 10mM DTT. cDNA was then synthesised with 2μL of Superscript II at 42°C for 1hour, followed by an additional 1μL of Superscript II for 2hours. Remaining RNA was hydrolysed with 3μL of 1M NaOH and 3μL of 0.5M EDTA at 65°C for 15min, then neutralised with 3μL of 1M HCl.

Purification of the cDNA to remove unincorporated aa-dUTPs was performed with a Millipore PCR cleanup kit according to manufacturer's instructions (Millipore, Bedford, Massachusetts). The micron PCR reservoir column was pre-washed with 500μL of sterile water by centrifugation at 1,000g for 9min to remove residual Tris which may bind the aa-dUTP and prevent Cy-dye conjugation. The sample was then mixed with 440μL of sterile water and loaded onto the column, before centrifugation for 16min at 2,000g. The membrane was washed with 450μL sterile water and re-spun and then exposed to 15μL of sterile water for 1min before the column was inverted and the sample eluted by centrifugation at 2,000g for 2min. This process was repeated with an additional 10μL of water, before the sample was dried by centrifugation under vacuum.

Labelling with Monofunctional Cy Dye and Hybridisation

The cDNA samples were then labelled with monofunctional Cy3 (reference population) and Cy5 (sample population) dyes (Amersham Biosciences, Cardiff, United Kingdom). Dried samples were resuspended in 4.5μL of fresh 0.1M sodium carbonate pH 9 and incubated at room temperature for 5min. To respective tubes, 4.5μL of Cy3 or Cy5 dye in DMSO was added and incubated in the dark for one hour, mixing every 15min. Unincorporated dye was then bound by 4.5μL of 4M hydroxylamine for 15min at which point it was possible to combine the Cy3/Cy5 samples. The labelled samples were then purified with a Qiagen PCR Clean-up kit according to manufacturer's instructions (Qiagen, Hilden, Germany). Briefly, the sample was added to 35μL of 100mM sodium acetate pH5.4 and 250μL PB Buffer, loaded to a column and centrifuged at 16,100g for 1min. The column was then washed with 750μL of buffer PE and spun for an additional minute to dry the column and the sample eluted with 2 aliquots of 30μL of EB buffer with a 5min incubation on the column in between. The efficiency of labelling was then determined for a single test run by examining absorbance at 260nm (detects total DNA), 550nm (detects unincorporated Cy3 dye) and 650nm (detects unincorporated Cy5 dye). To the above sample 10μg of Cot1 DNA (Invitrogen, Mt Waverly, Australia) and 20μg of poly dA (Roche Diagnostics, Basel, Switzerland) was added to final volume of 72μL to prevent nonspecific hybridisation to the chip. The probe was then concentrated to approximately 8μL via centrifugation under vacuum, and mixed with 6μL of 20x SSC, 15μL deionised formamide and 1.5μL of 10% sodium dodecyl sulphate (SDS). The sample was heated for 5min at 95°C to denature secondary structure, then Cot1 DNA and poly dA bound for an additional hour. A printed *O. scutellatus* venom gland microarray chip was then aligned in a hybridisation chamber and the sample loaded one quarter of the way up before covering with a glass coverslip. 10μL of water was added to each well in the hybridisation chamber where upon it was sealed and incubated at 45°C in the dark overnight to allow the Cy-dye labelled cDNA to hybridise to the transcripts on the chip.

Washing, Scanning and Analysis

The following day, hybridised slides were washed for 3 minutes in a solution containing 0.5% SDS and 0.2x SSC followed by two 3min washes in a second solution without SDS. Slides were then dried by centrifugation at 57g for 5min. The microarray chip was scanned on both

the Cy3 and Cy5 channels, taking multiple images at various gains to obtain the best exposure with a minimum number of spots that have exceeded 100% exposure. Scanning was performed with a GMS Array Scanner from Genetic Microsystems Inc. (Woburn, USA) with compatible software (version 1.51.0.42). Comparable images on both channels were then imported into Imagene analysis software and images overlayed (Biodiscovery Inc., El Sugndo, USA). Expression level data was then imported into Genespring 6 analysis software (Silicone Genetics, Forest Hill, Australia) with appropriate filters which was then used to examine varying levels of gene expression in the sample population of aRNA comparable to that of the *O. scutellatus* venom gland aRNA. In this manner it was possible to select a suit of genes of interest which could then be isolated from the original library and sequenced for further characterisation.

Array Screens: Conversion of λTripleEx2 to pTriplEx2

The conversion of λTripleEx2 clone with a desired insert to a pTripleEx2 plasmid involves the excision and circularisation of a complete plasmid from the recombinant phage by the Cre recombinase-mediated site-specific recombination at the *loxP* sites present within the clone by a bacterial host in which Cre recombinase is being expressed. Selected clones of interest from the results of the microarray hybridisations were isolated from the original phage preparations of the 4,800 recombinant cDNA library for sequence determination. Initially, a single colony of BM25.8 *E. coli* was used to inoculate 10mL of LB broth plus 10mM $MgSO_4$ and grown at 30°C overnight, the temperature at which Cre recombinase is expressed. The overnight culture was then inoculated with 100μL of 1M $MgCl_2$ and a 100μL aliquot of this culture preparation added to 75μL of each of the selected λphage in phage buffer. The mix was vortexed and incubated at 30°C for 30min, whereupon 200μL of LB broth was added and the sample incubated for a further 60min shaking at 30°C. The BM25.8 sample containing the converted plasmid was then streaked on an LB agar plate containing 100 μg/mL ampicillin and grown at 37°C overnight, and a colony selected for plasmid purification with a Qiagen miniprep kit as previously described. Alternatively the BM25.8 sample mixture was used to directly inoculate 4mL of LB broth plus ampicillin and this culture used for plasmid purification. Upon purification of the desired pTripleEx2 plasmid, the presence of an insert was confirmed via the previously described phage PCR method and then sequenced with either the 5'-LD forward or 3'-LD reverse primers. In this fashion a total of approximately

621 clones were selected from the taipan cDNA library from the results of the microarray analysis, converted to plasmid and subsequently sequenced.

Quantitative PCR Analysis

Genes of interest identified by screens of the *O. scutellatus* microarray chip were then analysed by quantitative PCR (qPCR). Quantitative PCR allows for the real-time analysis of relative mRNA expression levels of a particular gene, and was performed to confirm the expression results of the microarray chip. qPCR in the *O. scutellatus* and *O. microlepidotus* liver sample was performed on the list of genes in the table below (provided with the primer sequences used for amplification and size of product), along with the house-keeping gene required for standardisation, 60S ribosomal protein. qPCR was also performed in all snake venom gland RNA samples, as well as an *O. scutellatus* aRNA sample, for the calglandulin-like protein.

Gene	Size	Forward Primer Seq.	Reverse Primer Seq.
Calglandulin-like protein	199bp	5'-GAT CGG CAT CAA CCC CAC CAA-3'	5'-GTC CCA CTC AAT GTA GCC CTT G-3'
Pseudechetoxin-like protein	200bp	5'-GTG GGA AAA CTC CGT TGT GGT G-3'	5'-CAT TTG GCA GAA GCA CAA CCA-3'
Short α-Neurotoxin	173- 179bp	5'-TGA CCT TGG TGG TGG TGA CAA-3'	5'-AGG GCA ACC ACA TCC CCT TTC A-3'
Phospholipase A$_2$	192bp	5'-CCT GTC CAC CTT CTG GTC CTG T-3'	5'-TCT ACC GGT GTC CCG CTA CCT C-3
60S Ribosomal protein	195bp	5'-GCA AGC GTA TGA ACA CCA ACC C-3'	5'-AGA GCA GCT GGG ACG ACC ATT C-3'

Table 3.01. Genes analysed by quantitative PCR
Forward and reverse primer sequences and the predicted size of product for each gene analysed by qPCR are provided. Primers were designed within regions of the gene that were 100% identical between all snake species.

cDNA was synthesised for qPCR from 1.5μg of total RNA isolated from the venom glands of the 8 Australian elapids as previously described. cDNA was also synthesised from liver RNA purified from *O. scutellatus* and *O. microlepidotus* as well as from venom gland aRNA from

O. scutellatus. A 1:50 dilution of the cDNA was used in subsequent qPCR reactions as template. The qPCR reaction volumes were consistent with those previously described for the factor X and factor V-like genes, as were the cycling conditions. The only variation from this protocol was for the amplification of the pseudechetoxin-like protein, which used only 1.25ρmol of primer mix and an annealing temperature of 63°C. Duplicate runs of all experiments were performed. A standard curve of cycle time using a range of known quantities of plasmid as template was also performed. *O. scutellatus* calglandulin-like plasmid was used with appropriate primers at concentrations of 2,000ρg/μL decreasing by 10-fold serial dilutions to 0.2ρg/μL, producing a standard curve of cycle time for product formation which could be used to estimate the concentrations of a particular transcript in a sample.

PCR products were run on a 1% TAE agarose gel to confirm amplification of the right sized product and the results including cycle time for product formation and calculated transcript concentration as determined by the Rotor-Gene software exported into Microsoft Excel. Averages of the triplicate samples in duplicate runs were taken, and the results normalised to that of the house-keeping gene which was assumed to have equal expression in all tissue samples on the basis of the microarray results and the published literature.

Identification of Pseudechetoxin-like cDNAs

A dominant clone observed from sequence results of the microarray chip was that of a pseudechetoxin-like protein previously identified in *P. australis*. Primers were designed from the published 5'-UTR and 3'-UTR sequences of pseudechetoxin and were used in the amplification of a similar transcript in related species. PCR amplification of the full length, 717bp coding sequence of the gene was performed with approximately 500ng of cDNA template, 1 unit of AmpliTaq gold buffered in 10mM Tris-HCl pH 8.3, 50mM KCl, 2.25mM $MgCl_2$ and 200μM dNTPs with 50ρmol of each of the forward (5'-GGA GTT ACA CTG GGG CTC-3') and reverse (5'-ACT GAA TGG GAG ATC AGC-3') primers in a total volume of 50μL. The experiment was run with appropriate no template control.

The reaction mixture was then thermocycled with an initial denaturation at 95°C for 10min, followed by 35 cycles of 95°C for 30sec, 54°C for 45sec and 72°C for 1min, with a final

extension of 72°C for 7min. The resulting PCR product was analysed on a 1% TAE agarose gel stained with ethidium bromide. Bands of interest were excised, purified with a QIAex II gel extraction kit, ligated into pGEM-T, cloned and purified all as previously described. Multiple clones from each snake the pseudechetoxin-like protein was identified in were sequenced in the forward and reverse directions with T7 and M13 sequencing primers and alignments of the results performed with BioEdit software to determine to consensus sequence from each elapid. For phylogenetic analyses, the predicted protein sequences were aligned using ClustalW and subjected to pairwise deletion analysis using MEGA 2.1 software via the Neighbour-Joining method (1000 bootstrap replicates) (Kumar *et al.*, 2001). In this analyis, the *Gallus gallus* sequence XP420051 was defined as an outgroup, with the inclusion of a number of other venom CRISP (cysteine rich secretory protein) sequences for comparative analysis.

Identification of Calglandulin-like cDNAs

A clone demonstrating increased expression in the *O. scutellatus* venom gland identified by screens of the microarray chip originally proved unidentifiable by BLAST searches. Therefore this sequence was further characterised by 5'-RACE. Two 5'-RACE products were amplified from *O. scutellatus* and *P. textilis* 5'-RACE cDNA using two gene specific primers designed from the clone identified from the library: AR1 (5'-CTC TTA CCC CCT TCT TCT TGG TAT CGC CG-3') and AR2 (5'-GAC TCT TGC TGC GGA CCA GGG CCA TGA C-3'). Products were run on a 1% TAE agarose gel, purified for both snakes and subsequently cloned and sequenced. Sequence alignment results identified the protein as being highly homologous to a calglandulin-like protein previously cloned from *B. insularis*.

RACE (Rapid Amplification of cDNA Ends) provides a mechanism for generating full-length cDNAs in reverse transcription reactions using a single primer site. All 5'-RACE reactions in this study were performed with Clontech's (Palo Alto, California) SMART™ RACE cDNA Amplification kit. 5'-RACE ready cDNA was synthesised from RNA isolated from the venom gland of *O. scutellatus* and *P. textilis*. A total of 1μg of RNA was incubated in a final volume of 5μL with 5'-CDS primer (5'-(T)$_{25}$VN-3') at a final concentration of 2μM and SMART II A oligonucleotide (5'-AAG CAG TGG TAT CAA CGC AGA GTA CGC GGG-3') also at a final concentration of 2μM, at 70°C for 2min. cDNA was then synthesised with

200 units of Superscript II buffered in 50mM Tris-HCl pH 8.3, 3mM MgCl$_2$, 75mM KCl, 2mM DTT and 1mM of a dNTP mix to final reaction volume of 10μL, incubating at 42°C for 90min. The 5'-RACE ready cDNA was diluted in 100μL Tricine-EDTA buffer pH 8.5, heated to 72°C for 7min to inactivate the Superscript II and stored at -20°C awaiting use in RACE reactions. Each 50μL RACE reaction was composed of 1μL of Advantage 2 Polymerase mix buffered in 40mM Tricine-KOH pH 8.7, 15mM KOAc, 3.5mM Mg(OAc)$_2$, 375ng BSA, 0.005% Tween, 0.005% Nonidet P-40 and 0.2mM dNTP mix, amplifying with a Universal primer mix (40nM Long primer 5'-CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA ACT TCG CAG AGT-3' and 200nM Short primer 5'-CTA ATA CGA CTC ACT ATA GGG C-3') and 200nM of a gene specific primer. 2.5μL of 5'-RACE cDNA was added for each snake species being analysed and the whole reaction mixture thermocycled for 5 cycles at 94°C for 5sec then 72°C for 3min, followed by 5 cycles of 94°C for 5 sec, 70°C for 10sec and 68°C for 3min, followed by a final 20 cycles of 94°C for 5sec, 68°C for 10sec and 72°C for 3min.

The full-length coding sequence of the calglandulin-like gene was subsequently identified in the other Australian elapids by PCR. Amplification of an approximately 480bp PCR product was performed with forward (5'-CGA GGA AAT GGC AGCAAC ACT AAC-3') and reverse (5'-GTC TTA CTG AGT CAG TTT GAA GG-3') primers designed from the *B. insularis* sequence as follows. The reaction mixture contained approximately 200ng of cDNA template, 1 unit of AmpliTaq gold buffered in 10mM Tris-HCl pH 8.3, 50mM KCl, 1.75mM MgCl$_2$ and 200μM dNTPs with 25ρmol of each primer in a final volume of 25μL. The reaction was thermocycled at 95°C for 10min followed by 30 cycles of 95°C for 30sec, 56°C for 40sec and 72°C for 1min, with a final extension at 72°C for 7min. The PCR product was then run on a 1% TAE gel and purified, cloned and sequenced all as previously described. Alignments of the deduced amino acid sequence were then performed using BioEdit software along with phylogenetic analysis of the mRNA sequence using MEGA 2.1 software via the Neighbour-Joining method (1000 bootstrap replicates) (Kumar *et al.*, 2001).

Identification of Novel Phospholipase A$_2$ Toxins

A full-length phospholipase A$_2$ enzyme transcript coding for the *O. scutellatus* venom protein β-taipoxin was identified from screens of the microarray. Using primers designed from this

sequence, other phospholipase A$_2$ enzymes were identified from the venom glands of related snakes via PCR and cloning. The PCR amplification of the coding sequence of these genes was performed with approximately 200ng of venom gland cDNA template, 1 unit of AmpliTaq gold buffered in 10mM Tris-HCl pH 8.3, 50mM KCl, 1.8mM MgCl$_2$ and 200μM dNTPs with 25ρmol of each primer in a final volume of 25μL. Primer sequences for the amplification were forward (5'-TGC TTG CAG CTT CAC CAC TGA C-3) and reverse (5'-TCC TCG CGC TGA AGC CTC TCA AA-3). All reactions were then thermocycled at 95°C for 10min, then 12 cycles of 95°C for 30sec, 60°C for 30 sec and 72°C for 1min, another 13 cycles of 95°C for 30sec, 64°C for 30sec and 72°C for 1min with a final extension of 72°C for 7min. A total of 15μL of each PCR product was then run on a 1% TAE agarose gel stained with ethidium bromide and bands excised and cloned as previously described. A minimum of 12 clones were sequenced from each snake and alignments of the deduced amino acid sequence performed with BioEdit software.

Identification of L-Amino Acid Oxidase

A putative toxin, L-Amino Acid Oxidase (LAAO) has been previously identified in a number vipers. To search for the presence of a homologous gene in the venom glands of Australian elapids, PCR was performed using primers designed from an alignment of a number of these vipers. Amplification of a 1550bp product was performed with forward (5'-GAT GAA TGT CTT CTT TAT GTT CTC-3') and reverse (5'-TTA AAG TTC ATT GTC ATT GCT CA-3') primers which corresponded to the 5'- and 3'-coding sequences of the LAAO gene in *Trimeresurus stejnegeri* (AY338966), *Crotalus adamanteus* (AF071564), *Calloselasma rhodostoma* (AJ271725) and *Crotalus atrox* (AF093248). The 25μL PCR reaction mixture was prepared with approximately 400ng of fresh venom gland cDNA, 1 unit of AmpliTaq gold buffered in 10mM Tris-HCl pH 8.3, 50mM KCl, 2mM MgCl$_2$ and 200μM dNTPs with 25ρmol of each of the above primers. The reaction was thermocycled for 8min at 95°C, followed by 20 cycles of 95°C for 30sec, 53°C for 30sec and 72°C for 2min 30sec, and an additional 20 cycles of 95°C for 30sec, 55°C for 30sec and 72°C for 2min 30sec with a final extension of 72°C for 7min. PCR products were examined on a 1% TAE agarose gel, purified, cloned and sequenced in both directions all as previously described. Multiple clones from *O. scutellatus*, *P. australis* and *N. scutatus* were examined, and alignments of the deduced amino acid sequence performed.

Results

Generation of a cDNA Microarray from the Venom Gland of *O. scutellatus*

A cDNA microarray from the venom gland of the coastal taipan (*O. scutellatus*) was generated using the SMART™ cDNA library construction kit. An example of the *O. scutellatus* amplified cDNA is shown in figure 3.01A, lane 3. The titre of the amplified library was calculated to be approximately 2.5×10^6 pfu/mL with an average insert size of 200bp to 2kb. Sequence analysis of 136 clones randomly selected from the library revealed a redundancy of approximately 52%. Note that a similar library was produced from the venom gland of *P. textilis*, however this was not selected due to a greater redundancy in clone sequence. A cDNA microarray chip was then established with 4,800 PCR products of inserts amplified from random clones selected from the *O. scutellatus* library. Figure 3.01B shows the PCR products amplified from 192 clones selected for arraying on the chip. Note the variable size of the clones which, in this instance, range from 250bp to 1.5kb.

A.

B.
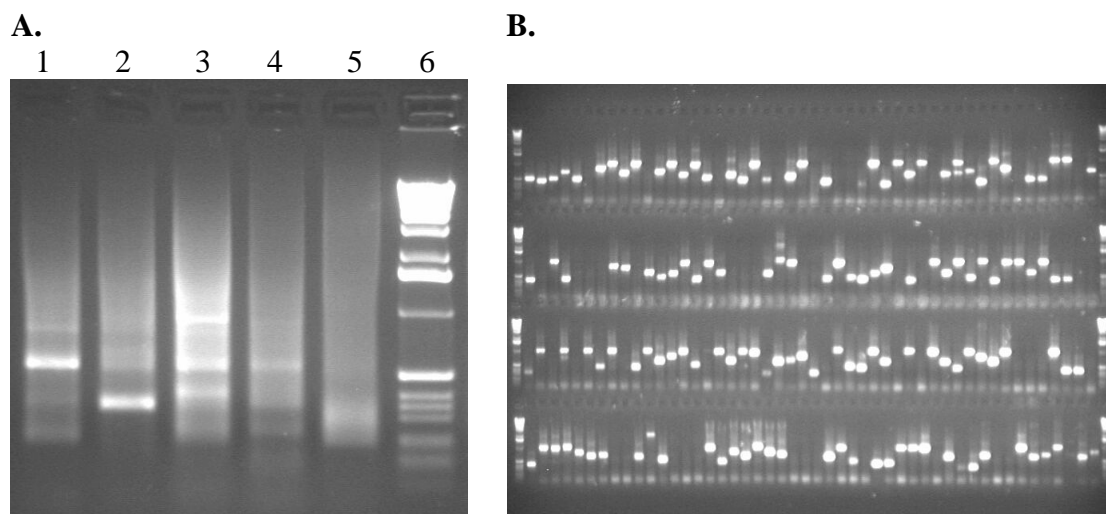
Figure 3.01. *O. scutellatus* cDNA library preparation.
(A) Amplification via long-distance PCR of venom gland cDNA from 1) *P. textilis*, 2) *P. porphyriacus*, 3) *O. scutellatus*, 4) *N. scutatus*, 5) *T. carinatus* and 6) Molecular weight marker. Note the presence of a single dominant band in the first two samples making them inadequate for the preparation of a representative cDNA library. *O. scutellatus* amplified cDNA was eventually selected for preparation of the microarray chip. **(B)** Example of 192 individual PCR products that were amplified from the *O. scutellatus* cDNA library and spotted onto the microarray chip.

Identification of Venom Gland Specific Transcripts

In order to identify proteins that may be of functional importance in the venom gland of the coastal taipan, a cross comparison of gene expression levels between the venom gland and the liver was performed, which would be expected to only overlap in the expression of house keeping genes, or those involved in typical cellular regulatory function. Total RNA isolated and amplified from both the venom gland and the liver of *O. scutellatus* were cross hybridised to the microarray chip to identify genes that were differentially expressed between these two tissues. All results were confirmed by performing similar comparisons with RNA isolated from the closely related species of snake, *O. microlepidotus*.

Following normalisation and filtering, an average of 57% of genes demonstrated an increase in expression in the venom gland compared to the liver. This is evident in figure 3.02 which shows that a large proportion of the genes arrayed on the chip are over-expressed in the venom gland (note the peak skewed below the 1 axis). To establish if these genes were specific to the venom gland, 582 transcripts were selected from those over-expressed in the venom gland for further analysis. Clones were identified by their position on the microarray grid, the original bacteriophage isolated and DNA fragments of interest recombined into a plasmid vector via a Cre-recombinase system for DNA sequencing. Thirty-nine clones that demonstrated increased expression in the liver were also selected for sequencing to gain an overall picture of the microarray chip, bringing the total number of sequenced transcripts to 621.

Using this approach, a total of 58 separate transcripts were identified from the *O. scutellatus* cDNA microarray chip (Genbank accession numbers DQ084027 to DQ084065 and DQ085813 to DQ085855). The results in table 3.02 demonstrates eight transcripts . corresponding to known toxins previously described from the venom of snakes. These include a pseudechetoxin-like protein, phospholipase A_2 enzymes, α-neurotoxins, a venom natriuretic peptide and a venom nerve growth factor. In addition to these known toxin genes, 18 transcripts were identified that were also over-expressed in the venom gland but code for proteins whose homology indicated a non-secretory/regulatory function within the venom gland (table 3.02). A number of other transcripts corresponding to non-secretory proteins that were down regulated in the venom gland compared to the liver were also identified, again predicted to be important for regular cellular maintenance. While many of the clones

demonstrated homology to known genes, a total of 24 of the 58 transcripts displayed no known homology after BLAST search analysis.
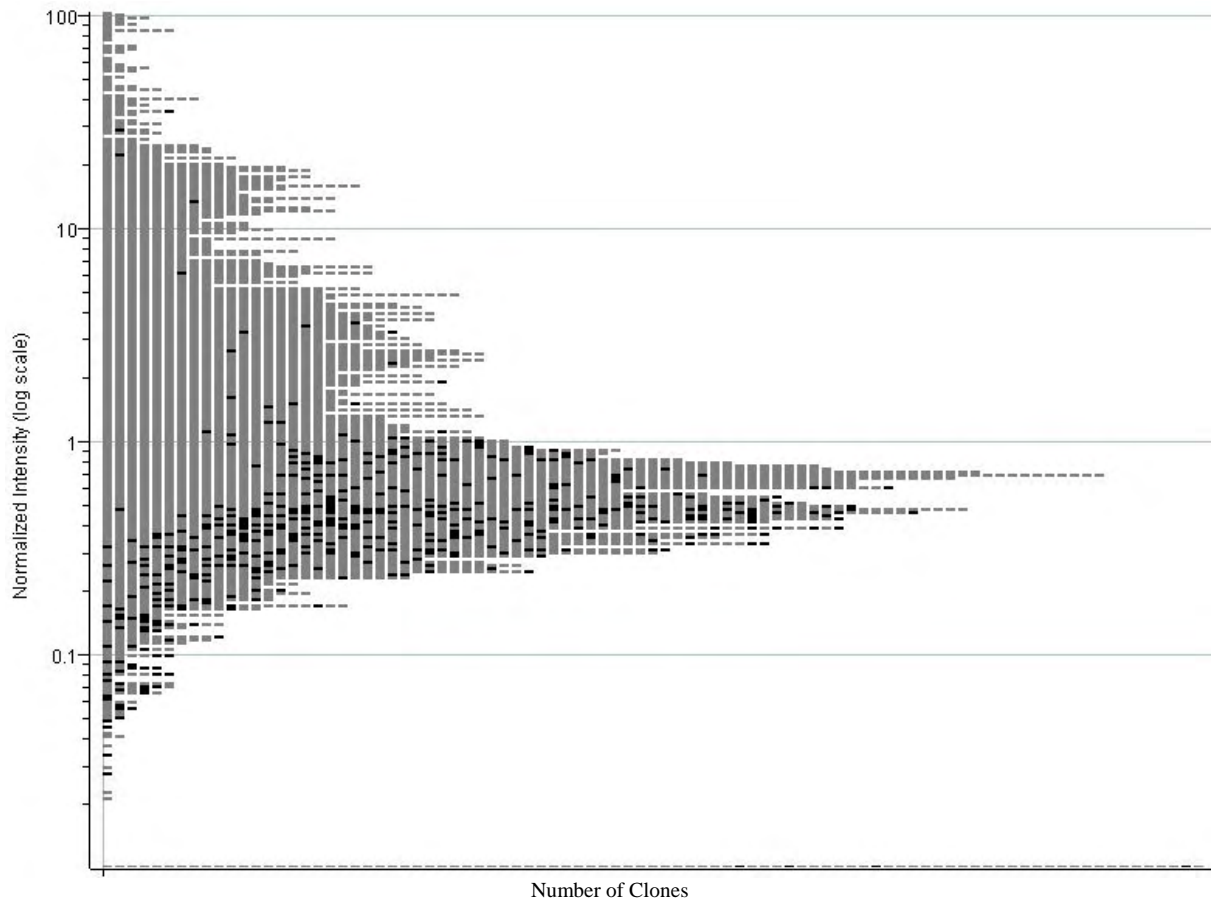
Figure 3.02. Histogram representing the normalised expression (in log scale) of all 4,800 transcripts present on the *O. scutellatu*s venom gland microarray chip.

The Y-axis denotes fold change between the two RNA populations: those above 1 demonstrate an increased expression in the liver, whilst those below 1 are more highly expressed in the venom gland. All known toxin and venom gland specific genes identified in screens of the library are highlighted in black. The skew below the 1 axis is due to the presence of a greater number of venom specific genes on the chip as the venom gland was the original source tissue for its production.

Name	# of clones	Insert size	Putative function	Express. ratio	Genbank accession #
Transcripts with increased venom gland expression					
Secreted/venom proteins					
Pseudechetoxin-like protein	191	1279bp	CNG ion channel blocker	+ 5.6	DQ084035
Phospholipase A_2 enzymes - 3 isoforms	53	760bp	Haemostatic, myotoxic and neurotoxic activities	+ 5.2	AY691657
α-Neurotoxins - 2 isoforms	22	536bp	Inhibitors of neuromuscular transmission	+ 12.4	DQ085855
Venom Natriuretic Peptide	1	498bp	Natriuresis and vasoactivity	+ 5.1	DQ084065
Venom Nerve Growth Factor	1	324bp	Undefined role in the venom	+ 3.4	DQ084064
Nonsecreted/regulatory proteins					
Calglandulin-like protein	217	469bp	Exportation of toxins into the venom	+ 2.4	DQ084027
Ribosomal Proteins* - 8 different forms	70	665bp	Ribosomal protein function	+1.8	DQ084040 - DQ084047
Creatine Kinase - 2 isoforms	5	688bp	Muscle derived enzyme	+ 5.5	DQ084048
Other proteins with known homology - 7 different proteins	13	381bp to 927bp	Proteins with known homology involved in regular cellular maintenance and activity	+2.2 to +99	DQ084054 - DQ084058, DQ084062, DQ084063,
Unknown transcripts - 8 different transcripts	9	167bp to 1022bp	Transcripts with no know or predicted homology	+1.2 to +44	
Transcripts with increased liver expression					
Proteasome homolog	4	619bp	Regulator of intracellular proteolytic cleavage	- 2.9	DQ084049
Polyadenylate binding protein homologue	1	1037bp	Required for translation initiation	- 2.8	DQ084050
Translocon associated protein (TRAP)-delta homologue	4	608bp	Transmembrane protein where nascent secretory proteins enter the ER	- 7.2	DQ084051
Other proteins with known homology - 5 different proteins	7	150p to 900bp	Proteins with known homology involved in regular cellular maintenance and activity	-3.7 to -11.7	DQ084052, DQ084053, DQ084059 - DQ084061
Unknown transcripts - 16 different transcripts	23	225bp to 1050bp	Transcripts with no know or predicted homology	-1.2 to -53.5	

Table 3.02. Transcripts identified from the *O. scutellatus* venom gland microarray.
A description for each transcript including total number of clones sequenced, average insert length, Genbank accession numbers and putative function as predicted by homology is included. The average fold difference in the expression levels of each gene between the venom gland and liver of *O. scutellatus* is also shown (+ values indicate increased venom gland expression, - values indicate increased expression in the liver). Note that expression levels shown for ribosomal proteins (*) are calculated for 60S ribosomal protein L13a only.

Of the toxin genes identified, three families of secreted proteins predominated. The great majority of these, 191 of 268 clones (71%), were represented by an *O. scutellatus* pseudechetoxin-like gene. Pseudechetoxin is a cyclic nucleotide gated ion channel blocker previously characterised from the venom of the mulga, *P. australis* (Brown *et al.*, 1999). Three different phospholipase A_2 isoforms were also identified, however the majority of these (51 of 53 clones) were represented by a full-length clone for the β-chain of taipoxin (Lind, 1982). Approximately 8% of the known toxin genes identified coded for short chain α-neurotoxins. Of the two isoforms identified, 20 of the 22 were a partial cDNA clone that showed 93% relatedness at the nucleotide level for a neurotoxin previously characterised from the venom of the marbled sea snake (*Aipysurus eydouxii*) (Li *et al.*, 2005). However translation of this sequence matched a neurotoxin previously purified and characterised at the native protein level from the coastal taipan (Zamudio *et al.*, 1996). The second isoform from the library had approximately 64% identity to the 3'-UTR an α-neurotoxin from the common brown snake (*P. textilis*) and hence would also be predicted to be a short chain neurotoxin (Gong *et al.*, 2000). A single clone was identified that represented the cDNA coding sequence of the full-length mature venom natriuretic peptide, a toxin with vasoactive properties from the inland taipan (*O. microlepidotus*). Translation of this clone revealed 88% homology to the mature protein sequence reported by Fry *et al.* (2005) and represents the first cDNA sequence of a natriuretic peptide from an Australian elapid. The final clone coded for a venom nerve growth factor and again represents the first description of such a factor in an Australian elapid snake. While the role a nerve growth factor may play in the venom is not immediately evident, it has previously been described in a number of other snakes (Kostiza and Meier, 1996). These include the many-banded krait, *Bungarus multicinctus*, whose mRNA sequence displayed 95% homology at the nucleotide level to the original clone identified from the coastal taipan microarray (Danse and Garnier, 1993).

The most frequently occurring transcript identified from screens of the microarray coded for a calglandulin-like protein that is related to the mammalian calcium binding protein calmodulin (Junqueira-de-Azevedo Ide *et al.*, 2003). This protein is not a toxin, but has been implicated in the export of toxins into the venom. To confirm the specificity of calglandulin and the afore mentioned toxins to the venom gland, quantitative PCR was performed with primers specific to the genes in the venom gland and the livers of both the inland and coastal taipan.

In all instances it was not possible to detect expression of these proteins within the liver of these snakes, confirming their venom gland specificity (results not shown).

To examine the relative expression of all known venom gland specific genes between elapid snakes, RNA isolated and amplified from the venom gland of these snakes was also hybridised to the microarray chip in duplicate runs. Table 3.03 depicts the average fold change of all clones from duplicate runs for the expression of each gene in each snake compared to that of *O. scutellatus*. Expression levels within the two liver samples and comparisons between *O. scutellatus* venom gland total RNA and amplified RNA are also shown.

	Pseudechetoxin-like protein	Calglandulin-like protein	PLA$_2$	Neurotoxin	Venom Natriuretic Peptide	Nerve Growth Factor
O. microlepidotus	-1.50	+2.42	+1.28	-1.11	-1.87	-1.27
P. textilis	-1.43	+2.89	-3.39	-17.25	-25.78	-2.31
N. scutatus	-1.04	-2.07	-1.97	-6.89	-7.13	-2.45
T. carinatus	-1.53	+1.49	-2.75	-2.58	-1.42	+1.17
P. australis	-1.47	+2.25	-2.03	-4.16	-5.87	+2.85
P. porphyriacus	-1.32	+1.49	-1.23	-4.04	-14.72	2.70
O. scutellatus liver	-5.58	-2.36	-5.16	-12.42	-5.10	-3.41
O. microlepidotus liver	-20.49	-2.73	-17.08	-35.33	-51.09	-13.06
O. scutellatus aRNA	+1.40	-1.04	-1.30	-1.03	-1.643	-1.95

Table 3.03. Fold difference in expression level of venom gland specific transcripts.
Comparisons are made between the Australian elapids relative to that of the coastal taipan (*O. scutellatus*). Positive values indicate an increase in expression of that transcript in that snake compared to *O. scutellatus* whilst a negative indicates a decrease in expression.

Characterisation of Pseudechetoxin-like cDNAs

A clone present in abundance on the microarray chip demonstrated significant homology to pseudechetoxin, a peptidic toxin that targets cyclic nucleotide-gated (CNG) ion channels isolated from the venom of the mulga (*P. australis*) (Brown *et al.*, 1999). Using primers

based of the published *P. australis* sequence, the full-length coding sequence of the pseudechetoxin-like cDNA was amplified from a total of seven Australian elapids including *P. australis* and *P. porphyriacu*s (figure 3.03). Note that no product was observed in *T. carinatus*, which complements the decreased expression of pseudechetoxin-like protein compared to the coastal taipan observed in the microarray hybridisation data for this snake species (table 3.03).
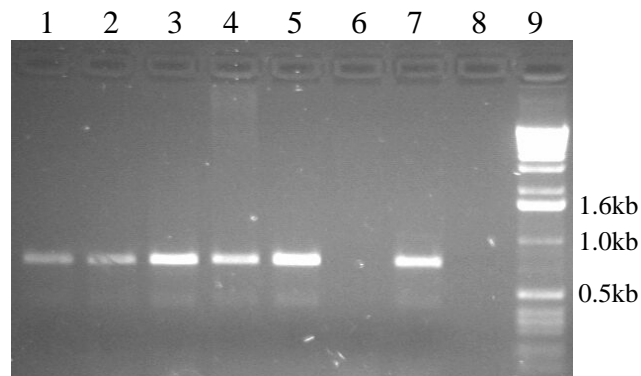
Figure 3.03. PCR amplification of the pseudechetoxin-like transcript.
A 768bp product was amplified from venom gland cDNA isolated from 1) *P. textilis*, 2) *P. porphyriacus*, 3) *O. scutellatus*, 4) *N. scutatus*, 5) *O. microlepidotus*, 6) *T. carinatus*, 7) *P. australis*, 8) no template control and 9) molecular weight marker. Note that a similar product was also identified from the venom of *H. stephensii* at a later date.

The results in figure 3.04 depict an alignment of the deduced protein sequence for the precursor toxin for all seven snakes. The unprocessed protein was 238 amino acids in length with an overall homology of 83% between the seven snakes. The sequences observed for *P. australis* and *P. porphyriacus* in this study were identical to those reported by Yamazaki *et al*. (2002). Pseudechetoxin is a member of the cysteine-rich secretory protein (CRISP) family of snake toxins, as is evidenced by a number of conserved cysteine residues, particularly within the C-terminal region of the protein (Jin *et al.*, 2003). A high degree of conservation between species is also observed within the propeptide sequence. This identity is particularly notable at the region surrounding the proposed cleavage site of the mature protein, despite the differences in the N-terminal region of the mature toxin reported by Yamazaki *et al*. (2002) between *P. australis* and *P. porphyriacus*.

```
                          Propeptide                    Mature protein
                            10         20         30         40         50         60
                       ....|....|....|....|....|....|....|....|....|....|....|....|
O. scutellatus      1  MIAFTVLLSLAAVLQQSSGTVDFASESSNKKDYRKEIVDKHNDLRRSVKPTARNMLQMKW  60
O. microlepidotus   1  MIAFIVLLSLAAVLQQSSGTVDFASESSNKKDYRKEIVDKHNDLRRSVKPTARNMLQMKW  60
P. textilis         1  MIAFLVLLSLAAVLQQSSGTVDFASESSNKNDYQKEIVDKHNDLRRSVKPTARNMLQMKW  60
N. scutatus         1  MIAFIVLLSLAAVLQQSSGTVDFASESSNKDYQKEIVDKHNALRRSVKPTARNMLRMEW   60
H. stephensii       1  MIAFIVLLSLAAVLQQSSGTVDFASESSNKKDYQKEIVDKHNALRRSVKPTARNMLRMKW  60
P. australis        1  MIAFIVLLSLAAVLQQSSGTADFASESSNKKNYQKEIVDKHNALRRSVKPTARNMLQMKW  60
P. porphyriacus     1  MIAFIVLLSLAAVLQQSSGTVDFASESSNKKNYQKEIVDKHNALRRSVKPTARNMLQMKW  60

                            70         80         90        100        110        120
                       ....|....|....|....|....|....|....|....|....|....|....|....|
O. scutellatus     61  NSRAAQNAKRWANRCTFAHSPPYTRTVGKLRCGENIFMSSQPFAWSGVVQAWYDEVKKFV 120
O. microlepidotus  61  NSRAAQNAKRWANRCTFAHSPPYTRTVGKLRCGENIFMSSQPFAWSGVVQAWYDEVKKFV 120
P. textilis        61  NSRAAQNAKRWANRCTFAHSPPYTRTVGKLRCGENIFMSSQPFAWSGVVQAWYDEVKKFV 120
N. scutatus        61  NSHAAQNAKRWADRCTFAHSPPHTRTVGKLRCGENIFMSSQPFAWSGVVQAWYDEVKKFV 120
H. stephensii      61  NSRAAQNAKRWADRCTFAHSPPHTRTVGKLRCGENIFMSSQPFAWSGVVQAWYDEVKKFV 120
P. australis       61  NSRAAQNAKRWANRCTFAHSPPNKRTVGKLRCGENIFMSSQPFPWSGVVQAWYDEIKNFV 120
P. porphyriacus    61  NSHAAQNAKRWADRCTFAHSPPNTRTVGKLRCGENIFMSSQPFPWSGVVQAWYDEIKNFV 120

                           130        140        150        160        170        180
                       ....|....|....|....|....|....|....|....|....|....|....|....|
O. scutellatus    121  YGIGAKPPSSVIGHYTQVVWYKSHLLGCASAKCSSTKYLYVCQYCPAGNIIGSIATPYKS 180
O. microlepidotus 121  YGIGAKPPSSVIGHYTQVVWYKSHLLGCASAKCSSTKYLYVCQYCPAGNILGSIATPYKS 180
P. textilis       121  YGIGAKPPSSVTGHYTQVVWYKSHLLGCASAKCSSTKYLYVCQYCPAGNIVGSIATPYKS 180
N. scutatus       121  YGIGAKPPGSVIGHYTQVVWYKSHLLGCASAKCSSTKYLYVCQYCPAGNIRGSIATPYKS 180
H. stephensii     121  YGIGAKPPGSVIGHYTQVVWYKSHLLGCASAKCSSTKYLYVCQYCPAGNIRGSIATPYKS 180
P. australis      121  YGIGAKPPGSVIGHYTQVVWYKSYLLGCASAKCSSSKYLYVCQYCPAGNIRGSIATPYKS 180
P. porphyriacus   121  YGIGAKPPGSVIGHYTQVVWYKSHLIGCASAKCSSSKYLYVCQYCPAGNIRGSIATPYKS 180

                           190        200        210        220        230
                       ....|....|....|....|....|....|....|....|....|....|....|...
O. scutellatus    181  GPPCGDCPSACDNGLCTNPCKHNNDFSNCKALAKKSKCQTEWIKSKCPATCFCRTEII 238
O. microlepidotus 181  GPPCGDCPSACDNGLCTNPCKHNDDLSNCKPLAKKSKCQTEWIKSKCPATCFCRTEII 238
P. textilis       181  GPPCGDCPSACDNGLCTNPCKHNDDLSNCKTLVKKHKCQTEWIKSKCPATCFCRTEII 238
N. scutatus       181  GPTCGDCPSACVNGLCTNPCKYEDDFSNCKALAKNSKCQTEWIKSKCPAACFCHNKII 238
H. stephensii     181  GPPCADCPSACVNGLCTNPCKHEDDFSNCKALAKNSKCQTAWIKSKCPATCFCHNKII 238
P. australis      181  GPPCADCPSACVNKLCTNPCKRNNDFSNCKSLAKKSKCQTEWIKKKCPASCFCHNKII 238
P. porphyriacus   181  GPPCADCPSACVNRLCTNPCNYNNDFSNCKSLAKKSKCQTEWIKKKCPASCFCHNKII 238
```

Figure 3.04. Alignment of pseudechetoxin-like proteins.

Comparisons are made between the deduced amino acid sequences from multiple cDNA clones from seven Australian elapid species. Putative propeptide and mature protein sequences are also shown with arrows. Genbank accession numbers are as follows: *O. scutellatus* DQ084035, *O. microlepidotus* DQ084036, *P. textilis* DQ084037, *N. scutatus* DQ084038 and *H. stephensii* DQ084039.

Phylogenetic analysis of the deduced amino acid sequence reveals a distinct clustering pattern, with high bootstrap support (figure 3.05). The two *Oxyuranus* genera associate closely with *P. textilis* with an overall identity of 96%, and likewise, *N. scutatus* and *H. stephensii* are most closely related to each other (97%). It is not surprising to observe the clustering of *P. porphyriacus* and *P. australis* as they arise from members of the same genus. This clustering pattern reflects the previous phylogenetic relationship of elapids determined on the basis of the factor X-like protease amino acid sequence described in chapter 2.
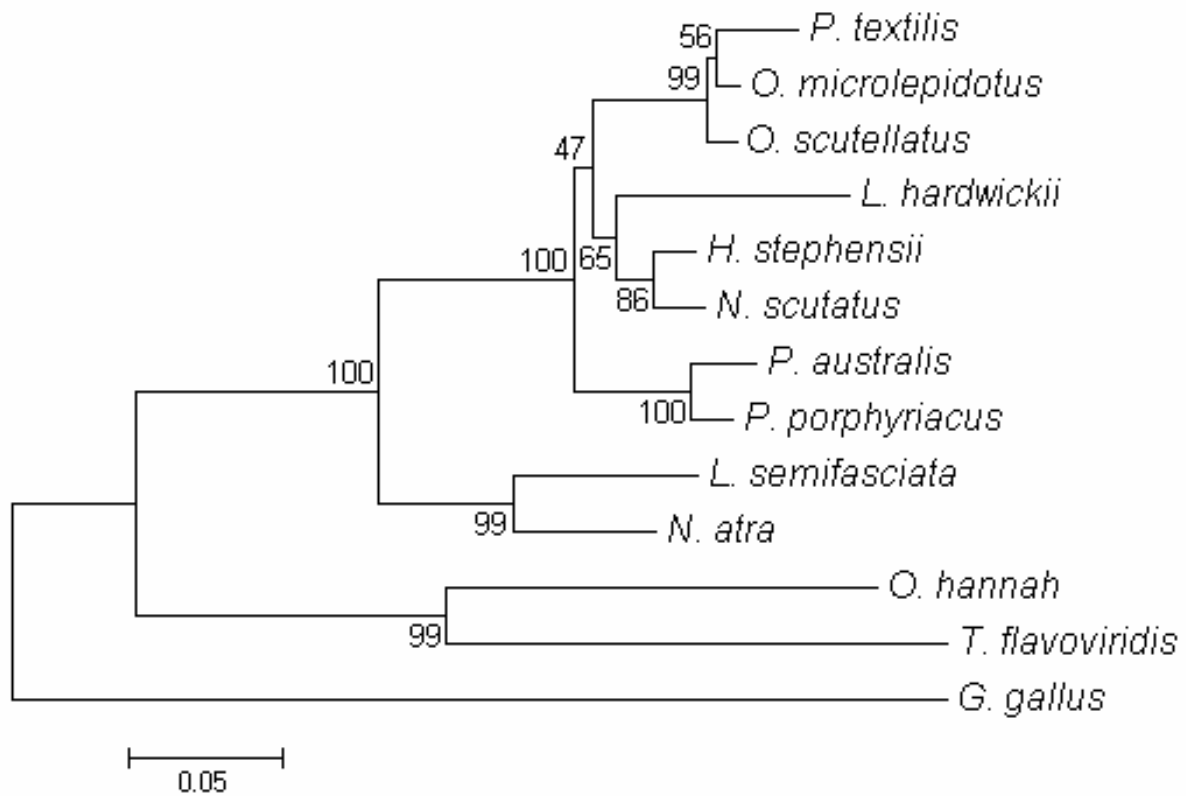
Figure 3.05. Phylogenetic relationship of pseudechetoxin-like proteins.
Analysis was performed using the neighbour-joining method with deduced protein sequences shown in figure 3.04 (Kumar *et al.*, 2001). Numbers above branches indicate the percentage of 1000 bootstrap replicates supporting the topology shown. The *Gallus gallus* sequence XP420051 was used as an outgroup with the incorporation of a number of other cysteine rich secretory protein sequences including *Lapemis hardwickii* (Q8UW25), *Laticauda semifasciata* (Q8JI38), *Naja atra* (AAP20603), *Ophiophagus hannah* (Q7ZT98) and *Trimeresurus flavoviridis* (Q8JI39) for phylogenetic comparison.

Characterisation of Calglandulin-like cDNAs

The most abundant clone identified from screens of the microarray chip represented a transcript corresponding to a calglandulin-like protein, an EF hand protein with conserved Ca^{2+} motifs first identified from the venom gland of the Island Jaracara, *Bothrops insularis* (Junqueira-de-Azevedo Ide *et al.*, 2003). The original clone isolated from the *O. scutellatus* cDNA library was a 465bp fragment that represented almost entirely the 3'-UTR region of the gene, however due to poor sequence homology, BLAST searches did not return a match.

Subsequently, 5'RACE was employed to identify the full-length transcript within *O. scutellatus* and *P. textilis*, identifying the clone as a calglandulin-like sequence (figure 3.06A). PCR primers designed from the *O. scutellatus* transcript were then employed to identify the full-length coding sequence in all the Australian elapids involved in this study (figure 3.06B).
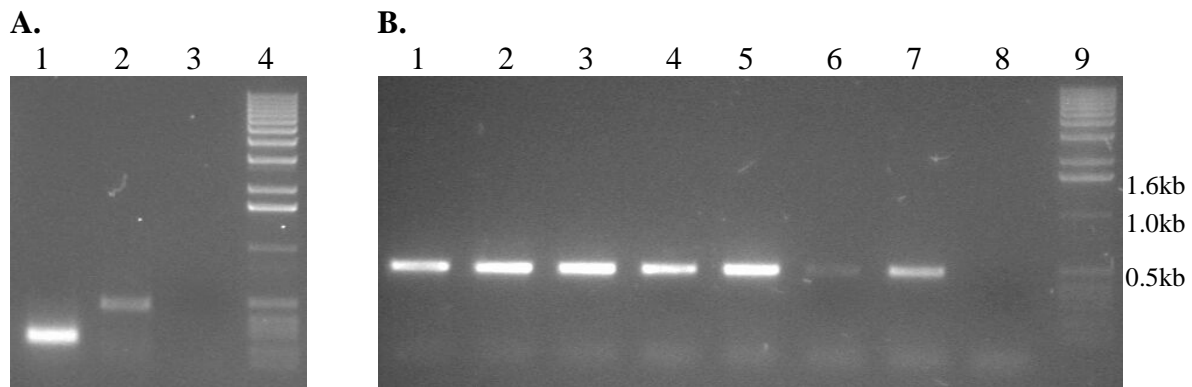
Figure 3.06. Identification of calglandulin-like transcripts.
(A) 5'-RACE amplification of a calglandulin-like gene from venom gland cDNA isolated from 1) *O. scutellatus* and 2) *P. textilis* along with 3) no template control and 4) and molecular weight marker. **(B)** PCR amplification of a 480bp product representing the full-length coding sequence of a calglandulin-like gene from venom gland cDNA isolated from 1) *O. scutellatus*, 2) *P. textilis*, 3) *O. microlepidotus*, 4) *P. porphyriacus*, 5) *P. australis* 6) *T. carinatus*, 7) *N. scutatus*, 8) no template control and 9) molecular weight marker. A similar product was also identified from *H. stephensii* at a later date.

The genes identified in all Australian elapids were designated the name CAGLP (calglandulin-like protein). Failure to originally identify the cDNA microarray clone as a calglandulin-like transcript was a result of the considerable difference observed between the entire 3'-UTR of the *B. insularis* and *O. scutellatus* sequences (only 54.9% homology where they overlap). Remarkably, there is a significant degree of identity (93.4%) across the 471 nucleotide open-reading frame (figure 3.07).

A full length CAGLP cDNA was observed in all eight Australian elapids. An alignment of the deduced amino acid sequences revealed complete conservation of this protein between all snakes with the exception of a glutamic acid in the place of an aspartic acid at residue 67 of *T. carinatus* (figure 3.08). A single nucleotide change at the third position explains this difference. This high degree of conservation was also maintained between calglandulin from

B. insularis where there were only three amino acid differences across the 156 residue protein. Although it was not possible to perform a phylogenetic analysis on the deduced protein sequence of the CAGLPs as a result of their high degree of identity, due to numerous silent substitutions in the nucleotide sequences, it was possible to examine such a relationship at the level of cDNA (figure 3.09). Again, clustering of the two *Oxyuranus* species with *P. textilis*, as well as the familiar grouping of the *Pseudechis* species together is evident. The cDNA sequence of calglandulin itself is most distantly related, not surprising as it arises from a viper, *B. insularis*. Again this phylogenetic relationship reflects that of the factor X-like protease and pseudechetoxin-like protein.


```
                                Start
O. scutellatus 1   -------ATGGCAGCAACACTAACCCCGGAGCAGATCACTGAATACAAAGGCATCTTTGAGATGTTTGACGAAGAAGGGA 73
B. jaracara    1   CGAGGAAATGGCAGCAACACTAACACCAGAGCAGATCACCGAATACAAAGGCATCTTTGAGATGTTTGATGAAGAAGGGA 80

O. scutellatus 74  ACGGCTTGGTGAAGACGGACGACTTGGAAAGCCTGATGAGTTTGATCGGCATCAACCCCACCAAGCGAGACCTCGCCAAC 153
B. jaracara    81  ACGGCTTGGTGAAGACAGATGATTTGGAAAGCCTCATGAGTCTGGTCGGGATCAACCCCACCAAGCGAGACCTGGCCAAC 160

O. scutellatus 154 ATGGCCAAGGACGTCGACAAAGACAAAAAAGGCACCTTCAACTGCGATGGGTTTCTGGTGTTGATGGGCATTTACCACGA 233
B. jaracara    161 ATGGCCAAGGATGTCGACAAAGACAAAAAAGGCACCTTCAATTGTGATGGGTTTCTGGCGTTGATGGGCATTTACCACGA 240

O. scutellatus 234 GAAATCCAAAAATCAGGACGAGGAACTCCGAGCCGCCTTTAAAGTCTTCGACAAAGAACACAAGGGCTACATTGAGTGGG 313
B. jaracara    241 GAAATCCAAAAATCAGGATGAGGAACTTAGAGCCGCCTTTAAAGTCTTCGACAAAGAACATAAGGGCTACATTGAGTGGG 320

O. scutellatus 314 ACACGCTCAAATACGTATTGATGAACGCCGGGGAACCGCTGAACGAACACGAAGCTGAACTCATGATGAAGGAAGCTGAC 393
B. jaracara    321 ACACGCTCAAGTACGTATTGATGAACGCCGGAGAACCCCTGAACGAACAGGAAGCTGAACTCATGATGAAGGAAGCAGAC 400

                                                                             Stop
O. scutellatus 394 AAAGATGGGGATGGGACCATCGACTACGAAGAATTTGTGGCCATGATGACGGGCGAATCCTTCAAACTGACTCAGTAAGA 473
B. jaracara    401 AACGACGGGGATGGGACCATTGACTACGAAGAATTTGTGGCCATGATGACGGGCGAGTCCTTCAAACTGACTCAATGAGA 480

O. scutellatus 474 CCTCCGCGAAACTTTTCGGCTTCGTCTCCCCTATCTTTGCTTTCTCTTTCTCCGGCGATACCAAGAAGAAGGGGGTAAGA 553
B. jaracara    481 C---------ACTTTTCGGTCGTCTCTCCTGCC--TGCTCCCTCTTCCTTCGACGATGCCAAGCAG----GGGTA--- 541

O. scutellatus 554 GACCGTGGCAGAACCTGGACGGCA-AATAACAACACGTAAAGTCATGGCCCTGGTCCGCAGCAAGAGTCTTTC--GTCAG 630
B. jaracara    541 -ACCGCGACGGAACCTGGACACCACAACAGCGGCCCGCA--GTCCCAGCCCTGGTACGCCGCGAGAGTCTTTTAAGCTGA 618

O. scutellatus 631 GATTCCAGCAATCGCCCTAATTAAATCACGATTCTCGAGC--CAAACTTCAAAAAACCTCAAAGGATTCATTACAGGCGA 708
B. jaracara    619 GGACGAAACAAAAGCCTTCTTGAGCTGAGACTGCGCGATTTTCAACCTTTAGTAGACCC----ACATTCGGAACACCCAA 694

O. scutellatus 709 CATACTGGCACTGCCCTGTATGAAGCCGACTCTGGCCTACATCATTTACAGTCCCCTGTGACCCTGTTTTCCCCTCCCCC 788
B. jaracara    695 TTTGTCCTCGCCGAGTTTTGTCAATAAAGCCCGGGTCAAATTTGAACACGCGAGGTTTTGGAAATG------------- 760

O. scutellatus 789 CAGGTTGGGTGTGTCATAAATCAGATTCTCCAATTGAGCACCTTCAAGGGCTGGGTGTCATCAATCACGTTCTCCAATTA 868
B. jaracara    760 -------------------------------------------------------------------------------- 760

O. scutellatus 869 AACCTATTCATGGGCAAAAAAAAAAAAAAAAAAAAAAAAAAAA 911
B. jaracara    760 ------------------------------------ 760
```

Figure 3.07. Alignment of the full-length cDNA sequences of calglandulin from *B. jaracara* and the calglandulin-like protein from *O. scutellatus*.

Note the high degree of homology within the coding sequence which drops of significantly in the 3'-UTR. Start and stop codons are marked by a box.

```
                          10        20        30        40        50        60
                    ....|....|....|....|....|....|....|....|....|....|....|....|
Calglandulin      1 MAATLTPEQITEYKGIFEMFDEEGNGLVKTDDLESLMSLVGINPTKRDLANMAKDVDKDK 60
O. scutellatus    1 MAATLTPEQITEYKGIFEMFDEEGNGLVKTDDLESLMSLIGINPTKRDLANMAKDVDKDK 60
O. microlepidotus 1 MAATLTPEQITEYKGIFEMFDEEGNGLVKTDDLESLMSLIGINPTKRDLANMAKDVDKDK 60
P. textilis       1 MAATLTPEQITEYKGIFEMFDEEGNGLVKTDDLESLMSLIGINPTKRDLANMAKDVDKDK 60
N. scutatus       1 MAATLTPEQITEYKGIFEMFDEEGNGLVKTDDLESLMSLIGINPTKRDLANMAKDVDKDK 60
T. carinatus      1 MAATLTPEQITEYKGIFEMFDEEGNGLVKTDDLESLMSLIGINPTKRDLANMAKDVDKDK 60
H. stephensii     1 MAATLTPEQITEYKGIFEMFDEEGNGLVKTDDLESLMSLIGINPTKRDLANMAKDVDKDK 60
P. australis      1 MAATLTPEQITEYKGIFEMFDEEGNGLVKTDDLESLMSLIGINPTKRDLANMAKDVDKDK 60
P. porphyriacus   1 MAATLTPEQITEYKGIFEMFDEEGNGLVKTDDLESLMSLIGINPTKRDLANMAKDVDKDK 60

                          70        80        90       100       110       120
                    ....|....|....|....|....|....|....|....|....|....|....|....|
Calglandulin     61 KGTFNCDGFLALMGIYHEKSKNQDEELRAAFKVFDKEHKGYIEWDTLKYVLMNAGEPLNE 120
O. scutellatus   61 KGTFNCDGFLVLMGIYHEKSKNQDEELRAAFKVFDKEHKGYIEWDTLKYVLMNAGEPLNE 120
O. microlepidotus 61 KGTFNCDGFLVLMGIYHEKSKNQDEELRAAFKVFDKEHKGYIEWDTLKYVLMNAGEPLNE 120
P. textilis      61 KGTFNCDGFLVLMGIYHEKSKNQDEELRAAFKVFDKEHKGYIEWDTLKYVLMNAGEPLNE 120
N. scutatus      61 KGTFNCDGFLVLMGIYHEKSKNQDEELRAAFKVFDKEHKGYIEWDTLKYVLMNAGEPLNE 120
T. carinatus     61 KGTFNCEGFLVLMGIYHEKSKNQDEELRAAFKVFDKEHKGYIEWDTLKYVLMNAGEPLNE 120
H. stephensii    61 KGTFNCDGFLVLMGIYHEKSKNQDEELRAAFKVFDKEHKGYIEWDTLKYVLMNAGEPLNE 120
P. australis     61 KGTFNCDGFLVLMGIYHEKSKNQDEELRAAFKVFDKEHKGYIEWDTLKYVLMNAGEPLNE 120
P. porphyriacus  61 KGTFNCDGFLVLMGIYHEKSKNQDEELRAAFKVFDKEHKGYIEWDTLKYVLMNAGEPLNE 120

                         130       140       150
                    ....|....|....|....|....|....|....|.
Calglandulin    121 QEAELMMKEADKDGDGTIDYEEFVAMMTGESFKLTQ 156
O. scutellatus  121 HEAELMMKEADKDGDGTIDYEEFVAMMTGESFKLTQ 156 DQ084027
O. microlepidotus 121 HEAELMMKEADKDGDGTIDYEEFVAMMTGESFKLTQ 156 DQ084028
P. textilis     121 HEAELMMKEADKDGDGTIDYEEFVAMMTGESFKLTQ 156 DQ084029
N. scutatus     121 HEAELMMKEADKDGDGTIDYEEFVAMMTGESFKLTQ 156 DQ084030
T. carinatus    121 HEAELMMKEADKDGDGTIDYEEFVAMMTGESFKLTQ 156 DQ084031
H. stephensii   121 HEAELMMKEADKDGDGTIDYEEFVAMMTGESFKLTQ 156 DQ084032
P. australis    121 HEAELMMKEADKDGDGTIDYEEFVAMMTGESFKLTQ 156 DQ084033
P. porphyriacus 121 HEAELMMKEADKDGDGTIDYEEFVAMMTGESFKLTQ 156 DQ084034
```

Figure 3.08. Alignment of calglandulin-like proteins (CALGP).

Comparisons were performed with the deduced amino acid sequence from multiple cDNA clones of the calglandulin-like protein identified in all Australian elapid species involved in this study, compared with calglandulin from *B. jaracara*. Genbank accession numbers are provided at the end of each sequence.

To determine the relative expression of CAGLP within the venom gland, the microarray chip was hybridised with RNA isolated from the Australian elapids. Calglandulin was selected for such a cross-species comparison due to its presence and significant sequence identity in all species involved. The average expression of this transcript compared to that of *O. scutellatus* was determined in duplicate RNA hybridisations to the microarray chip and confirmed via qPCR analysis (figure 3.10). It is evident from both the array and qPCR results that CAGLP is not expressed in the liver of either *Oxyuranus* species, confirming observations by Junqueira-de-Azevedo *et al*. (2003) that calglandulin is a venom gland specific protein. Expression of the CAGLP gene varied between Australian snakes, with the greatest amount present within *P. textilis*, whilst *N. scutatus* had the lowest level of transcription. No bias in experimental results was observed as a result of amplifying the RNA used in hybridisations, confirming the validity of its use in microarray experiments (figure 3.10).
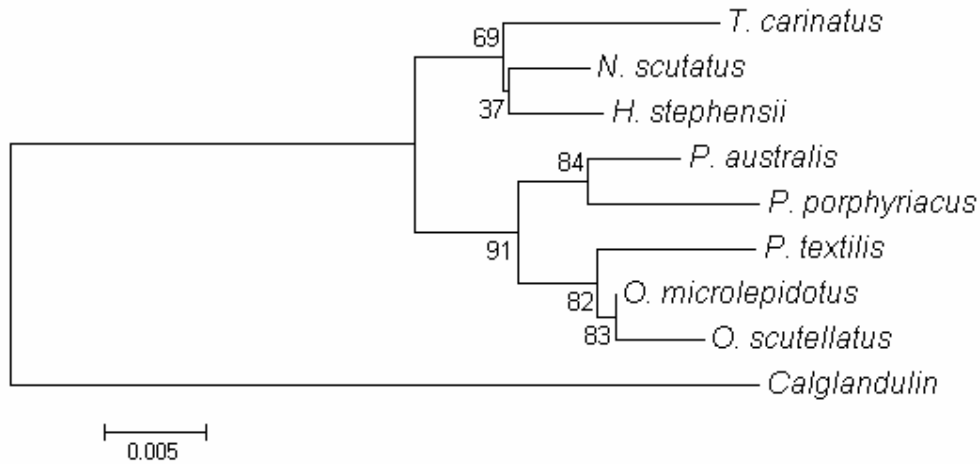
Figure 3.09. Phylogenetic relationship of the calglandulin-like cDNAs.

Note that the phylogenetic analysis of calglandulin-like transcripts resembles that of the other toxins identified in this study.
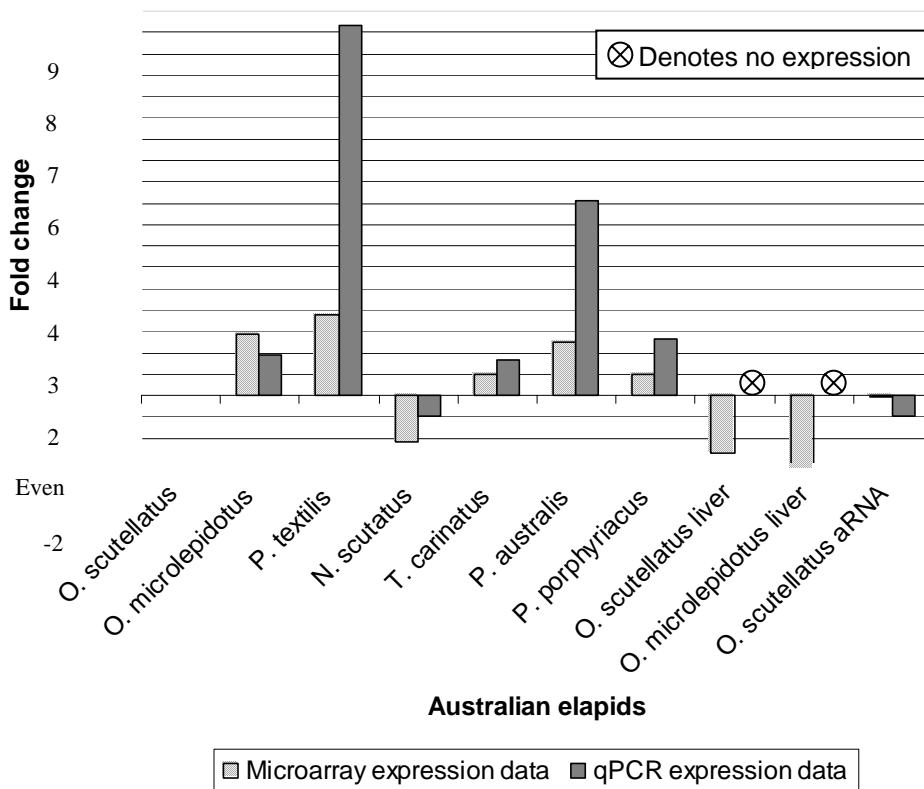
Figure 3.10. Graph comparing expression of the calglandulin-like genes.

Expression ratios shown are relative to that of the coastal taipan (*O. scutellatus*), determined by hybridisation of aRNA to the *O. scutellatus* microarray chip and via quantitative PCR. Note the absence of expression of this gene within the liver of the *Oxyuranus* genera, and the relative even expression when comparing total RNA with amplified RNA. Positive values denote a relative increase in expression in that species.

<u>Characterisation of Phospholipase A$_2$ cDNAs</u>

The third most abundant type of clone detected from screens of the *O. scutellatus* microarray were phospholipase A$_2$ (PLA$_2$) enzymes. One isoform, corresponding to the full-length mRNA transcript of the β-chain of taipoxin with a 438bp open reading frame, 92bp 5'-UTR and 230bp 3'UTR, represented the majority of the PLA$_2$ clones identified from the microarray chip. Taipoxin is a potently active toxin within the venom of the coastal taipan and is composed of three PLA$_2$ chains: α, β and γ (Fohlman *et al.*, 1976). Evidence from other Australian elapids, including *P. australis*, *N. scutatus* and the lowland copperhead *Austrelaps superbus*, indicates that there are in fact multiple PLA$_2$ variants present within the venom of any one snake (Singh *et al.*, 2000; Francis *et al.*, 1995; Takasaki *et al.*, 1990). In order to investigate the presence of other phospholipase A$_2$ enzymes within the venom of *O. scutellatus*, primers were designed within the 5'- and 3'-UTR of the β-taipoxin clone to amplify potential isoforms from venom gland cDNA via PCR. DNA sequencing of isolated clones provided evidence for at least eight separate proteins, including the original β-chain of taipoxin. The results in figure 3.11 demonstrate an alignment of the deduced amino acid sequence of these variants. Note there may be yet other PLA$_2$s present within the venom of *O. scutellatus* not detected by these primer pairs.

Overall there was 46.1% homology between the precursor proteins from the venom gland of the coastal taipan with complete conservation of cysteine residues involved in putative disulfide bond formation (figure 3.11). Two of the clones identified corresponded to the full-length coding sequence of β-taipoxin with the exception of a single amino acid change within a highly conserved signal peptide of 27 residues length. Two additional clones (designated OS-1 and OS-3) equated to the mature protein sequence of the previously characterised PLA$_2$ OS-1 described by Lambeau *et al*. (1995). The identified clones OS-6 and OS-7 demonstrated significant homology to the previously described α-chain of taipoxin and the mature protein sequence of OS-2 respectively, differing by only a few amino acids. The N-terminal region (amino acids 1-33 of the mature protein sequence without propeptide) of the final two clones, OS-4 and OS-5, were identical to each other, but different to all other clones in this region. However for the remaining 86 to 94 amino acids, there was little sequence similarity between these two clones. Interestingly OS-4 differed by only a single amino acid compared to OS-1 in this region, while OS-5 was identical to OS-2 over the C-terminal

portion of the protein. The multitude of PLA$_2$ genes described here for *O. scutellatus* and demonstrated previously for other snake species is likely to have arisen by gene duplication and recombination events (Moura-da-Silva *et al.*, 1995). It is likely that such a recombination event was responsible for the generation of the PLA$_2$ clones OS-4 and OS-5.

```
                              Signal peptide            Mature protein
                              10        20         30        40        50        60
                         ....|....|....|....|....|....|....|....|....|....|....|....|
Beta taipoxin          1 MHPAHLLVLLAVCVSLLGASDIPPLPLNLVQFGKMIECAIRNRRPALDFMNYGCYCGKGG 60
Beta taipoxin variant1 1 MHPAHLLVLSAVCVSLLGASDIPPLPLNLVQFGKMIECAIRNRRPALDFMNYGCYCGKGG 60
O. scutellatus OS-5    1 MHPAHLLVLLAVCVSLLGASDIPPLPLNLVQFGFMIECAIRNRQPALDFMNYGCYCGTVG 60
O. scutellatus OS-4    1 MHPAHLLVLLAVCVSLLGASDIPPLPLNLVQFGFMIECAIRNRQPALDFMNYGCYCGTVG 60
O. scutellatus OS-1    1 MHPAHLLVLLAVCVSLLGAARIPPLPLSLLNFANLIECANHGTRSALAYADYGCYCGKGG 60
O. scutellatus OS-3    1 MHPAHLLVLLAVCVSLLGAARIPPLPLSLLNFANLIECANHGTRSALAYADYGCYCGKGG 60
O. scutellatus OS-6    1 MHPAHLLVLLAVCVSLLGASDIPPLPLNLLQFGYMIRCANRRTRPVWHYMDYGCYCGKGG 60
O. scutellatus OS-7    1 MHPAHLLVLLAVCVSLLGASDIPPLPLNLAQFGFMIRCANGGSRSPLDYTDYGCYCGKGG 60
OS-2                   1 -------------------------NLAQFGFMIRCANGGSRSPLDYTDYGCYCGKGG 33
Alpha taipoxin         1 -------------------------NLLQFGFMIRCANRRSRPVWHYMDYGCYCGKGG 33

                              70        80        90        100       110       120
                         ....|....|....|....|....|....|....|....|....|....|....|....|
Beta taipoxin         61 SGTPVDDLDRCCQVHDECYAEAEK------HGCYPSLTTYTWECRQVGPYCN-SKTQCEV 113
Beta taipoxin variant1 61 SGTPVDDLDRCCQVHDECYAEAEK------HGCYPSLTTYTWECRQVGPYCN-SKTQCEV 113
O. scutellatus OS-5   61 CGTPVDDLDRCCQVHDECYGEAEKR-----LGCSPFVTLYSWKCYGKAPSCN-TKTDCQR 114
O. scutellatus OS-4   61 RGTPLDDLDRCCHVHDDCYGEAEKLPACNYLMSSPYFNSYSYKCNEGKVTCTDDNDECKA 120
O. scutellatus OS-1   61 RGTPLDDLDRCCHVHDDCYGEAEKLPACNYLMSSPYFNSYSYKCNEGKVTCTDDNDECKA 120
O. scutellatus OS-3   61 RGTPLDDLDRCCQVHDDCYGEAEKLPACNYLMSSPYFNSYSYKCNEGKVTCTDDNDECKA 120
O. scutellatus OS-6   61 SGTPVDDLDRCCQVHDECYGEAKRR-----FRCAPYWTLYSWKCYGTAPSCN-TKTQCEG 114
O. scutellatus OS-7   61 SGTPVDDLDRCCQVHDECYGEAEKR-----LGCSPFVTLYSWKCYGKAPSCN-TKTDCQR 114
OS-2                  34 RGTPVDDLDRCCQVHDECYGEAEKR-----LGCSPFVTLYSWKCYGKAPSCN-TKTDCQR 87
Alpha taipoxin       34 SGTPVDDLDRCCQVHDECYGEAVRR-----FGCAPYWTLYSWKCYGKAPTCN-TKTRCQR 87

                              130       140       150
                         ....|....|....|....|....|....|....|
Beta taipoxin        114 FVCACDFAAAKCFAQEDYNPAHSNINTGER--CK 145 AY691657
Beta taipoxin variant1 114 FVCACDFAAAKCFAQEDYNPAHSNINTGER--CK 145 DQ085813
O. scutellatus OS-5  115 FVCNCDAKAAECFARSPYQKKNWNINTKAR--CK 146 DQ085817
O. scutellatus OS-4  121 FICNCDRTAAICFAGATYNDENFMISKKRNDICQ 154 DQ085816
O. scutellatus OS-1  121 FICNCDRTAAICFAGATYNDENFMISKKRNDICQ 154 DQ085814
O. scutellatus OS-3  121 FICNCDRTAAICFAGATYNDENFMISKKRNDICQ 154 DQ085815
O. scutellatus OS-6  115 FVCNCDAKAAECFARSPYQPSNRNINTKER--CK 146 DQ085818
O. scutellatus OS-7  115 FVCNCDAKAAECFARSPYQKKNWNINTKAR--CK 146 DQ085819
OS-2                  88 FVCNCDAKAAECFARSPYQKKNWNINTKAR--CK 119 AAB33760
Alpha taipoxin       88 FVCRCDAKAAECFARSPYQNSNWNINTKAR--CR 119 P00614
```

Figure 3.11. Alignment of all coastal taipan (*O. scutellatus*) PLA$_2$ clones.
The deduced amino acid sequence of all PLA$_2$ clones identified from the coastal taipan compared to previously published sequences OS-2 and alpha taipoxin is shown. Note that OS-6 and OS-7 demonstrate significant homology to alpha taipoxin and OS-2 respectively. Conserved cysteine residues predicted to be involved in disulfide bond formation are shaded gray. Genbank accession numbers are included at the end of the sequence.

Using the same PCR primer pair, a similar approach was employed to identify phospholipase cDNAs from other Australian elapids (figure 3.12). Note that no product was observed in *H. stephensii*, but is probably indicative of difficulty in primer annealing during PCR as opposed

to an absence of transcript in this snake. Subsequent cloning and sequencing of at least a dozen clones from this PCR product identified multiple phospholipase variants in each snake. The results in figure 3.13 depict an alignment of phospholipase proteins deduced from cDNA clones identified in all Australian elapids. All clones identified, with the exception of PT-PLA1 (AF082983) from *P. textilis* and NS-1 (X14043) from *N. scutatus*, represent novel sequences at the cDNA level. The predicted amino acid sequences for most of these clones have also not been previously described at the protein level. The clones listed in figure 3.13 are just a representative group of distinct isoforms identified from each snake; in many cases there were several other clones documented that differed by only a small number of amino acids from the variants shown.
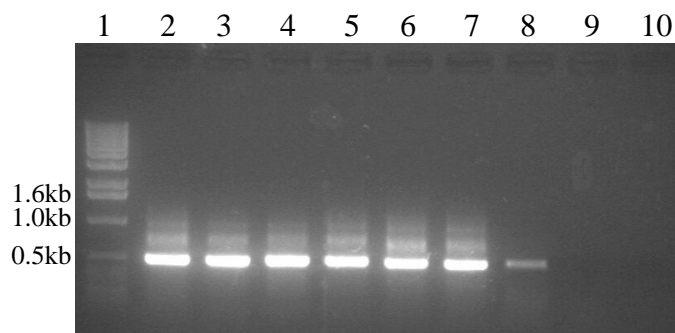
Figure 3.12. PCR amplification of PLA$_2$ transcripts.

A 488bp product representing the full length cDNA transcript of PLA$_2$ enzymes was amplified from the venom glands of 1) molecular weight marker, 2) *O. scutellatus*, 3) *O. microlepidotus*, 4) *P. textilis*, 5) *N. scutatus*, 6) *P. porphyriacus*, 7) *P. australis*, 8) *T. carinatus*, 9) *H. stephensii* and 10) no template control.

Amongst the PLA$_2$ clones identified from the venom gland of *O. scutellatus* was the β-chain of taipoxin, whose protein sequence has been previously characterised from the venom of this snake. Of the six different phospholipase A$_2$ isoforms identified from the closely related species *O. microlepidotus*, one demonstrated 85% identity to β-taipoxin. This clone has been designated the name β-paradoxin-like because of its probable identity with a β-taipoxin homolog known to be present within the venom of this snake (Fohlman, 1979). Even though the protein sequences of any of the paradoxin chains have not been determined, a purified form has demonstrated presynaptic neurotoxic activity similar to that of taipoxin (Bell *et al.*, 1998). It is of interest that the other PLA$_2$ clones from *O. microlepidotus* as a group are most closely related to those of *O. scutellatus*.

```
                          Signal peptide        Mature protein    Ca2+ binding      Active site
                            10        20        30        40        50        60        70        80
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
O. scutellatus OS-5       MHPAHLLVLLAVCVSLLGASDIPPLPLNLVQFGFMIECAIRNRQPALDFMNYGCYCGTVGCGTPVDDLDRCCQVHDECYG 80
O. scutellatus OS-4       MHPAHLLVLLAVCVSLLGASDIPPLPLNLVQFGFMIECAIRNRQPALDFMNYGCYCGTVGRGTPLDDLDRCCHVHDDCYG 80
O. scutellatus OS-1       MHPAHLLVLLAVCVSLLGAARIPPLPLSLLNFANLIECANHGTRSALAYADYGCYCGKGGRGTPLDDLDRCCHVHDDCYG 80
O. scutellatus OS-6       MHPAHLLVLLAVCVSLLGASDIPPLPLNLLQFGYMICANRRTRPVWHYMDYGCYCGKGGSGTPVDDLDRCCQVHDECYG 80
O. scutellatus OS-7       MHPAHLLVLLAVCVSLLGASDIPPLPLNLAQFGFMIRCANGGSRSPLDYTDYGCYCGKGGSGTPVDDLDRCCQVHDECYG 80
Beta taipoxin             MHPAHLLVLLAVCVSLLGASDIPPLPLNLVQFGFMIECAIRNRRPALDFMNYGCYCGTVGCGTPVDDLDRCCQVHDDCYG 80
O. microlepidotus OM-1    MHPAHLLVLLAVCVSLLGAARIPPLPLSLLDFANLIECANHGTRSALAYVHYGCYCGKGGRGTPVDELDRCCVHDDCYG 80
O. microlepidotus OM-2    MHPAHLLVLLAVCVSLLGASDIPPLPLNLAQFGFMIKCANGGSRPVSHYMDYGCYCGKGGSGTPVDELDRCCQVHDECYG 80
Beta paradoxin            MHPAHLLVLLAVCVSLLGAARIPPLPLNLLQFGFMIECAIRNRQPALDFMNYGCYCGTVGHGTPVDDLDRCCKTRNECYA 80
P. textilis Pt-PLA1       MHPAHLLVLLAVCVSLLGAARIPPLPLSLVEFRILIKCANHNSRNVLDYADYGCYCGKGGSGTPVDDLDRCCQAHDYCYD 80
Textilotoxin C            MHPAHLLVLLGVYVSLLGAARIPPLPLNLIQFSNMIKCTIPGSQPLLDYANYGCYCGPGNNGTPVDDVDRCCQAHDECYD 80
Textilotoxin D            MHPAHLLVLLGVCVSLLGAASIPRPSLNIMLFGNMIQCTIPCEQSWLGYLDYGCYCGSGSSGIPVDDVDKCCKTHDECYY 80
N. scutatus NS-2          MYPAHLLVLLAVCVSLLGAASIPARPLNLYQFGNMIQCANHGRRPARHYMDYGCYCGKGGSGTPVDELDRCCQTHDDCYG 80
N. scutatus NS-3          MYPAHLLVLLAVCVSLLGAASIPPLPLNLIQFDNMIECANYGRRPSWHYMDYGCYCGKGGSGTPVDELDRCCQTHDDCYG 80
N. scutatus NS-1          MYPAHLLVLLAVCVSLLGAASIPPLPLNLAQFDNMIECANYGSRPSWHYMDYGCYCGKEGSGTPVDELDRCCKTYDDCYT 80
N. scutatus NS-4          MYPAHLLVLLAVCVSLLGAASIPPLPLNLAQFDMLIQCANHHSRATWHYMDYGCYCGWGGSGTPVDDLLMCCKIHDDCYA 80
Scutoxin                  MYPAHLLVLLAVCVSLLGAASIPPQPLNLVQFSYLIQCANHGRRPTWHYMDYGCYCGAGGSGTPVDELDRCCKIHDDCYD 80
T. carinatus TC-1         MYPAHLLVLLAVCVSLLGAASIPARPLNLYQFGNMIECANHGRRPTRHYMDYGCYCGKGGSGTPVDELDRCCQTHDDCYG 80
T. carinatus TC-7         MYPAHLLVLLAVCVSLLGASDIRQQPLNLLQFAFMIRCANNRRRPVWHYMNYGCYCGKGGSGTPVDELDRCCKIHDDCYA 80
P. australis PA-16        MSPAHLLVLLAVCVSLLGASDVRPRPLNLIQFGNMIQCANKGSRPTRHYMDYGCYCGWGGSGTPVDELDRCCQTHDNCYG 80
P. australis PA-19        MSPAHLLVLLAVCVSLLGASDVRPRPLNLIQFGNMIQCANKGSRPTRHYMDYGCYCGWGGSGTPVDELDRCCQVHDNCYE 80
P. australis PA-20        MSPAHLLVLLAVCVSLLGASDVRPQPLNLILQFRKMIQCANKGSRAAWHYLDYGCYCGEGGRGTPVDELDRCCKIHDDCYI 80
P. australis PA-21        MSPAHLLVLLAVCVSLLGASDVRPQPLNLIQLSNMIKCAIPGSRPLFQYTDYGCYCGKGGHGTPVDELDRCCKAHDDCYG 80
P. porphyriacus PP-1      MSPAHLLVLLAVCVSLLGASDIRPRPLNLIQFGNMIQCANKGSRPTRHYMDYGCYCGPGGRGTPVDELDRCCQTHDNCYG 80
P. porphyriacus PP-3      MSPAHLLVLLAVCVSLLGASDIPPQPLNLYQFKNMIQCANKGSRATWHYLDYGCYCGPGGRGTPVDELDRCCQTHDNCYD 80
P. porphyriacus PP-6      MSPAHLLVLLAVCVSLLGASDIPPQPLNLYQFKNMIQCANKGSRSWLDYVNYGCYCGWGGSGTPVDELDRCCQTHDNCYG 80

                             90        100       110       120       130       140       150
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....
O. scutellatus OS-5       EAEKRLGGSPFVL-----LYSWKCYGKAPSC-NTKTDQRFVCNCDAKAAECFARSPYQKKNWNINTKAR--CK 146
O. scutellatus OS-4       EAEKLPACNYLMSSPYFNSYSYKCNEGKVTCTDDNDECKAFIQNCDRTAAICFAGATYNDENFMISKKRNDICQ 154
O. scutellatus OS-1       EAEKLPACNYLMSSPYFNSYSYKCNEGKVTCTDDNDECKAFIQNCDRTAAICFAGATYNDENFMISKKRNDICQ 154
O. scutellatus OS-6       EAKRRFRCAPYWL-----LYSWKCYGTAPSC-NTKTQCEGFVCNCDAKAAECFARSPYQPSNRNINTKER--CK 146
O. scutellatus OS-7       EAEKLPACNYLMSSPYFNSYSYKCNEGKVTCTDDNDECKAFIQNCDRTAAICFAGATYNDENFMISKKRNDICQ 154
Beta taipoxin             EAEKH-GCYPSLL-----TYTWECRQVGPYC-NSKTQCEVFVCNCDFAAAKCFAQEDYNPAHSNINIGER--CK 145
O. microlepidotus OM-1    EAEKLPACNYLMSSPYFNSYSYKCNEGKVTCTDDNDECKAFIQNCDRTAAICFAGATYNDENFMISKKRNDICQ 154
O. microlepidotus OM-2    EAEKRFKCVPYML-----LYSWKCYGTAPSC-NTKTDCQRFVCNCDAKAAECFARSPYQNKNWNINTKAR--CK 146
Beta paradoxin            EAEKH-GCYPSLL-----TYRWQCGRVGLHC-NSKTQCEVFVCNCDLAAAKCFAQEDYNPAHFNINTKAR--CK 145
P. textilis Pt-PLA1       DAEKLPACNYRFSGPYWNPYSYKCNEGEVTCTDDNDECKAFIQNCDRTAAICFAGAPYNDENFMITTKKKNICQ 154
Textilotoxin C            EASNH-GCYPELL-----LYDYYCDTGVPYC-KARTQCQVFVCGCDLAVAKCLAGATYNDENKNINTGER--CQ 145
Textilotoxin D            KAGQIPGCSVQPNEVFNVDYSYECNEGQLTCNESSNNECEMAVCNCDRAAAICFARFPYNKNYWSINIEIH--CR 152
N. scutatus NS-2          EAEKLPACNYLMSGPYYNTSYEHCNEGELTCKDNNDECKAFIQNCDRTAAICFARAPYNDANWNIDTKTR--C- 151
N. scutatus NS-3          EAEKLPACNYLMSGPYYNTSYEHCNEGELTCKDNNDECKAFIQNCDRTAAICFARAPYNDANWNIDTKTR--C- 151
N. scutatus NS-1          EAEKR-GCHPKFS-----AYSWKCGSDGPTC-DSETGCKRSVCDCDATAAKCSAKAPENQANWNIDTETH--CQ 145
N. scutatus NS-4          KAESS-SCNPKWL-----MYDYYCNSEGPYCRNIKKKCQRSVCDCDVEAAFCFARTPYNNSNWDIDTETH--CQ 146
Scutoxin                  EACKK-GCFPKMS-----AYDYYCGENGPYCRNIEKKCLRFVCDCDVEAAFCFAKAPYNNANWNIDTKKR--C- 146
T. carinatus TC-1         EAEKLPACNYMMSGPYYNTSYECNDGELTCKDNNDECKAFIQNCDRTAAICFARTPYNDANWNIDTKTR--C- 151
T. carinatus TC-7         RAIKSYSCAPYWL-----LYSWKCYGNGPTC-NSKTRQRFVCDCDSKAAMCFARAPYNNANWNIDTKKR--CR 146
P. australis PA-16        EAEKK-GCYPKLL-----LYSWDCTGNVPIC-SPKAECKDFVCACDABAAKCFAKAAYNDANWNIDTKTR--C- 144
P. australis PA-19        QACKK-GCFPKLL-----LYGWKCTGNVPTC-NPKTECKDFTCACDABAAKCFAKAPYKKENWNIDTKTR--CK 145
P. australis PA-20        EACKD-GCYPKLL-----WYSWDCTGDAPTC-NPKSKCKDFVCACDAAAAKCFAKAPYNKANWNIDTKTR--CK 145
P. australis PA-21        EACKK-GCYPVLL-----LYSWECTEKVPTC-NSKTKCKRIVCDCDAAVAKCFAKAPYKKENYNIDTKTR--CK 145
P. porphyriacus PP-1      EABKK-GCYPKML-----LYSWQCTNNVPTC-DPKTECKDFVCACDABAAKCFAKAAYNDANWNINTEKR--C- 144
P. porphyriacus PP-3      QACKK-GCFPKLL-----LYSWQCTGNVPTC-NSKSGCKDFVCACDABAAKCFAKAPYKKENFKIDTKTR--CR 145
P. porphyriacus PP-6      EAEKK-GCYPKML-----LYSWQCTNNVPTC-DPKTECKDFVCACDABAAKCFAKAAYNDANWNINTEKR--C- 144
```
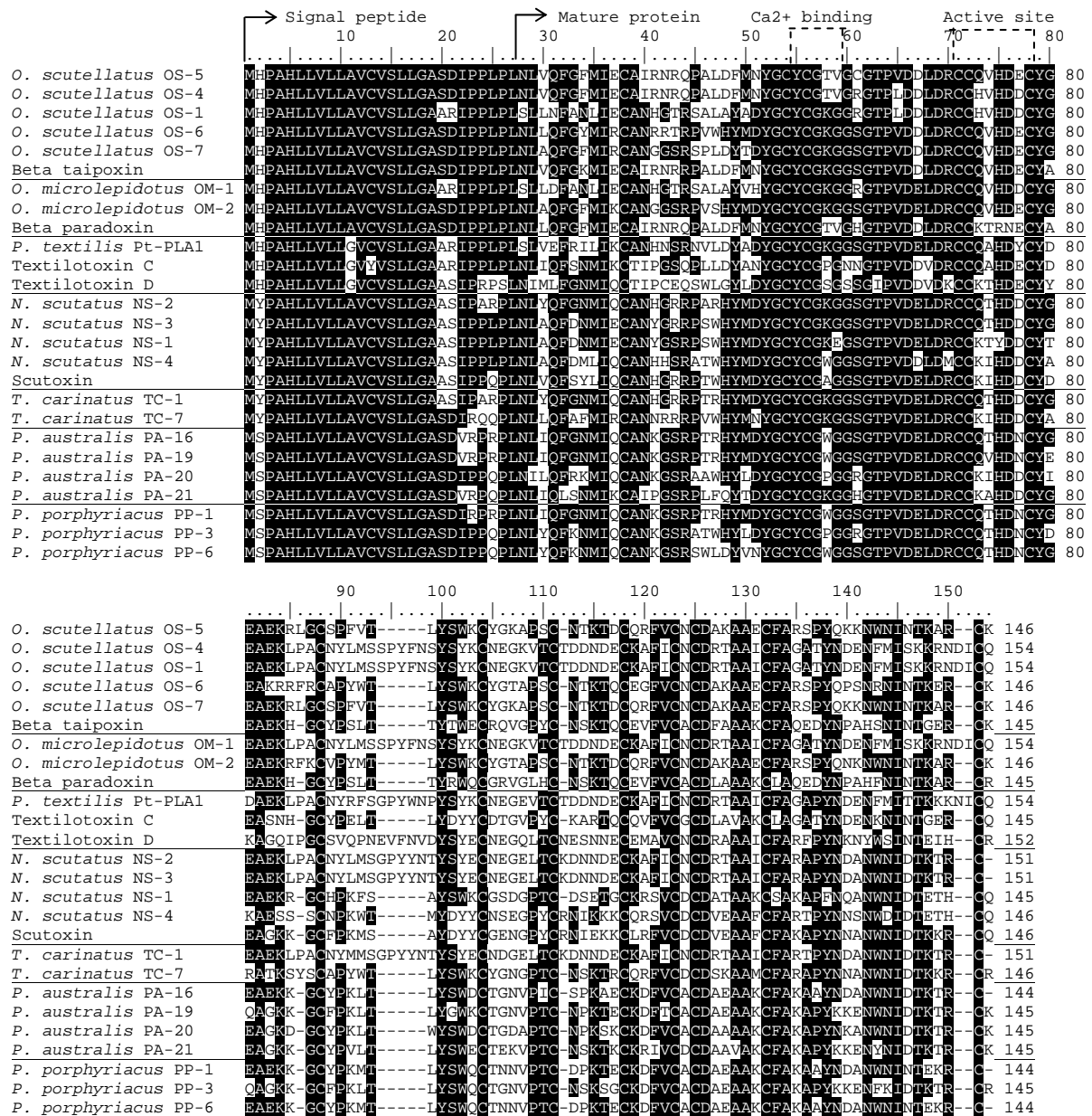
Figure 3.13. Protein sequence alignment of phospholipase A₂ enzymes.

Sequences were deduced from cDNA clones identified from the venom glands of seven Australian elapids (proteins from individual species are separated by lines). All sequences with the exception of *P. textilis* Pt-PLA1 and *N. scutatus* NS-1 represent novel clones at the level of cDNA. The signal peptide sequence, Ca^{2+} binding site and active site are shown. Where proteins have previously been identified, prior nomenclature is applied, otherwise novel PLA₂s are identified via a numbering system. All unique sequences and their variants (not shown) identified in this study are accessible via the Genbank database (DQ085813 to DQ085854).

Two clones identified from *P. textilis* had deduced amino sequences identical to the previously reported protein sequence for the C and D chains of textilotoxin (Pearson *et al.*, 1993). Textilotoxin is a noncovalently bound, multimeric protein complex composed of five phospholipase A_2 chains, A, B, C and two D chains that is a highly potent presynaptic blocker of neuromuscular transmission (Su *et al.*, 1983). Other novel PLA_2 cDNA sequences identified in this study that have previously been characterised at the protein level include scutoxin from *N. scutatus*, however in most instances the other PLA_2 clones represent their first description at either the protein or nucleotide level (Francis *et al.*, 1991). Indeed, for *T. carinatus* and *O. microlepidotus*, the above sequences represent the first description of any phospholipase from the venom gland of these snakes.

While there is considerable variation between the 26 sequences listed in figure 3.13 it is evident that the 27 amino acid signal peptide is highly conserved, as well as a block of sequence between residues 52 to 84 of the precursor protein. Interestingly, this conserved sequence contains both the Ca^{2+} binding loop (residues 55 to59 of the precursor protein, which has a conserved motif W/YCGxG in PLA_2s) and phospholipase enzymatic active site (residues 71 to 78 of the precursor protein, which has previously been shown to contain the conserved motif CCxxHDxC in other PLA_2s) (Arni and Ward, 1996). Not surprisingly, many of the pharmacological sites associated with various activities observed between different PLA_2s, for example, neurotoxicity, myotoxicity and anticoagulant effects, have been mapped to the less conserved C-terminal region of the protein (Kini, 2003). Fourteen cysteine residues previously shown to be involved in disulfide linkages within PLA_2s are also completely conserved amongst all species (Fry, 1999).

Characterisation of L-Amino Acid Oxidase cDNAs

The L-amino acid oxidase (LAAO) family convert L-amino acids into keto acids, ammonia and hydrogen peroxide, the later product proving to be a potent bactericide. It is speculated that LAAOs present within the venom help aid against putrification of the prey during the long period of digestion within the snake which may be contaminated with pathogens such as *Aeromonas* (Thomas and Pough, 1979). With the knowledge that LAAOs exist at least in the mulga, *P. australis*, and that there appears to be significant sequence identity between Australian elapids and other snake genera, primers were designed from a number of viper LAAOs to identify homologous proteins (Stiles *et al.*, 1991).
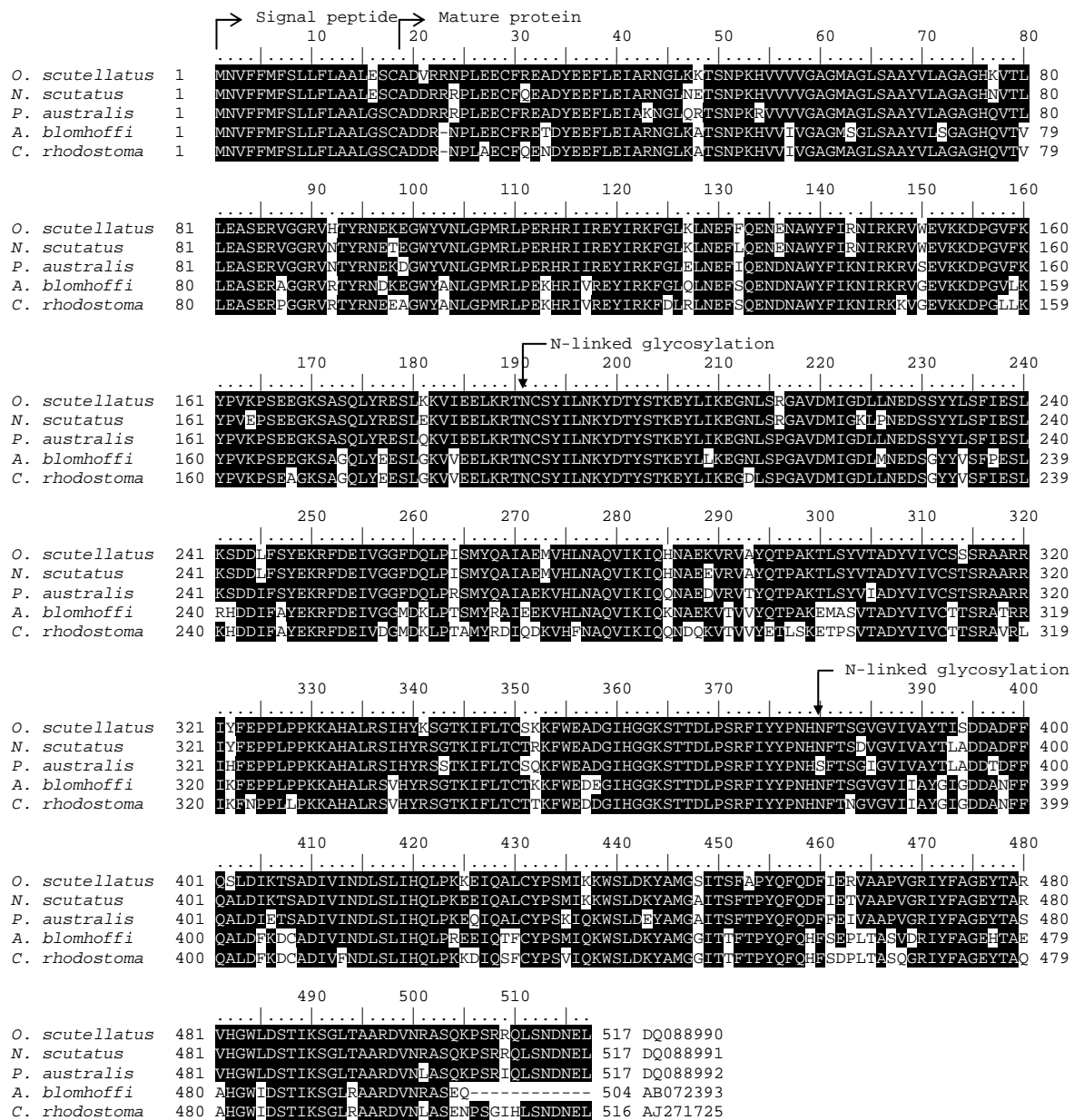
```
                                  Signal peptide     Mature protein
                          10          20        30        40        50        60        70        80
                          |    .    |    .    |    .    |    .    |    .    |    .    |    .    |    .    |
O. scutellatus    1  MNVFFMFSLLFLAALESCADVRRNPLEECFREADYEEFLEIARNGLKKTSNPKHVVVVGAGMAGLSAAYVLAGAGHKVTL   80
N. scutatus       1  MNVFFMFSLLFLAALESCADDRRPLEECFQEADYEEFLEIARNGLNETSNPKHVVVVGAGMAGLSAAYVLAGAGHNVTL   80
P. australis      1  MNVFFMFSLLFLAALGSCADDRRRPLEECFREADYEEFLEIAKNGLQRTSNPKRVVVVGAGMAGLSAAYVLAGAGHQVTL   80
A. blomhoffi      1  MNVFFMFSLLFLAALGSCADDR-NPLEECFRETDYEEFLEIARNGLKATSNPKHVVIVGAGMSGLSAAYVLSGAGHQVTV   79
C. rhodostoma     1  MNVFFMFSLLFLAALGSCADDR-NPLAECFQENDYEEFLEIARNGLKATSNPKHVVIVGAGMAGLSAAYVLAGAGHQVTV   79

                          90         100       110       120       130       140       150       160
                          |    .    |    .    |    .    |    .    |    .    |    .    |    .    |    .    |
O. scutellatus   81  LEASERVGGRVHTYRNEKEGWYVNLGPMRLPERHRIIREYIRKFGLKLNEFFQENBNAWYFIRNIRKRVWEVKKDPGVFK  160
N. scutatus      81  LEASERVGGRVNTYRNETEGWYVNLGPMRLPERHRIIREYIRKFGLKLNEFLQENBNAWYFIRNIRKRVWEVKKDPGVFK  160
P. australis     81  LEASERVGGRVNTYRNEKDGWYVNLGPMRLPERHRIIREYIRKFGLELNEFIQENDNAWYFIKNIRKRVSEVKKDPGVFK  160
A. blomhoffi     80  LEASERAGGRVRTYRNDKEGWYANLGPMRLPEKHRIVREYIRKFGLQLNEFSQENDNAWYFIKNIRKRVGEVKKDPGVLK  159
C. rhodostoma    80  LEASERPGGRVRTYRNEEAGWYANLGPMRLPEKHRIVREYIRKFDLRLNEFSQENDNAWYFIKNIRKKVGEVKKDPGLLK  159

                                                      N-linked glycosylation
                          170        180       190↓      200       210       220       230       240
                          |    .    |    .    |    .    |    .    |    .    |    .    |    .    |    .    |
O. scutellatus  161  YPVKPSEEGKSASQLYRESLKKVIEELKRTNCSYILNKYDTYSTKEYLIKEGNLSRGAVDMIGDLLNEDSSYYLSFIESL  240
N. scutatus     161  YPVEPSEEGKSASQLYRESLEKVIEELKRTNCSYILNKYDTYSTKEYLIKEGNLSRGAVDMIGKLPNEDSSYYLSFIESL  240
P. australis    161  YPVKPSEEGKSASQLYRESLQKVIEELKRTNCSYILNKYDTYSTKEYLIKEGNLSPGAVDMIGDLLNEDSSYYLSFIESL  240
A. blomhoffi    160  YPVKPSEEGKSAGQLYEESLCKVVEELKRTNCSYILNKYDTYSTKEYLLKEGNLSPGAVDMIGDLMNEDSGYYVSFPESL  239
C. rhodostoma   160  YPVKPSEAGKSAGQLYEESLGKVVEELKRTNCSYILNKYDTYSTKEYLIKEGDLSPGAVDMIGDLLNEDSGYYVSFIESL  239

                          250        260       270       280       290       300       310       320
                          |    .    |    .    |    .    |    .    |    .    |    .    |    .    |    .    |
O. scutellatus  241  KSDDLFSYEKRFDEIVGGFDQLPISMYQAIAEMVHLNAQVIKIQHNAEKVRVAYQTPAKTLSYVTADYVIVCSSRAARR  320
N. scutatus     241  KSDDLFSYEKRFDEIVGGFDQLPISMYQAIAEMVHLNAQVIKIQHNAEEVRVAYQTPAKTLSYVTADYVIVCSTSRAARR  320
P. australis    241  KSDDIFSYEKRFDEIVGGFDQLPRSMYQAIAEKVHLNAQVIKIQQNAEDVRVTYQTPAKTLSYVIADYVIVCSTSRAARR  320
A. blomhoffi    240  RHDDIFAYEKRFDEIVGGMDKLPTSMYRAIEEKVHLNAQVIKIQKNAEKVTVVYQTPAKEMASVTADYVIVCTTSRATRR  319
C. rhodostoma   240  KHDDIFAYEKRFDEIVDGMDKLPTAMYRDIQDKVHFNAQVIKIQQNDQKVTVVYETLSKETPSVTADYVIVCTTSRAVRL  319

                                                                          N-linked glycosylation
                          330        340       350       360       370       380↓      390       400
                          |    .    |    .    |    .    |    .    |    .    |    .    |    .    |    .    |
O. scutellatus  321  TYFEPPLPPKKAHALRSIHYKSGTKIFLTCSKKFWEADGIHGGKSTTDLPSRFIYYPNHNFTSGVGVIVAYTISDDADFF  400
N. scutatus     321  IYFEPPLPPKKAHALRSIHYRSGTKIFLTCTRKFWEADGIHGGKSTTDLPSRFIYYPNHNFTSDVGVIVAYTLADDADFF  400
P. australis    321  IHFEPPLPPKKAHALRSIHYRSSTKIFLTCSQKFWEADGIHGGKSTTDLPSRFIYYPNHSFTSGIGVIVAYTLADDTDFF  400
A. blomhoffi    320  IKFEPPLPPKKAHALRSVHYRSGTKIFLTCTKKFWEDFGIHGGKSTTDLPSRFIYYPNHNFTSGVGVIIAYGIGDDANFF  399
C. rhodostoma   320  IKFNPPLLPKKAHALRSVHYRSGTKIFLTCTTKFWEDDGIHGGKSTTDLPSRFIYYPNHNFTNGVGVIIAYGIGDDANFF  399

                          410        420       430       440       450       460       470       480
                          |    .    |    .    |    .    |    .    |    .    |    .    |    .    |    .    |
O. scutellatus  401  QSLDIKTSADIVINDLSLIHQLPKKEIQALCYPSMIKKWSLDKYAMGSITSFAPYQFQDFIERVAAPVGRIYFAGEYTAR  480
N. scutatus     401  QALDIKTSADIVINDLSLIHQLPKEEIQALCYPSMIKKWSLDKYAMGAITSFTPYQFQDFIETVAAPVGRIYFAGEYTAR  480
P. australis    401  QALDIETSADIVINDLSLIHQLPKEQIQALCYPSKIQKWSLDEYAMGAITSFTPYQFQDFFEIVAAPVGRIYFAGEYTAS  480
A. blomhoffi    400  QALDFKDCADIVINDLSLIHQLPREEIQTFCYPSMIQKWSLDKYAMGGITTFTPYQFQHFSEPLTASVDRIYFAGEHTAE  479
C. rhodostoma   400  QALDFKDCADIVFNDLSLIHQLPKKDIQSFCYPSVIQKWSLDKYAMGGITTFTPYQFQHFSDPLTASQGRIYFAGEYTAQ  479

                          490        500       510
                          |    .    |    .    |    .    |    .
O. scutellatus  481  VHGWLDSTIKSGLTAARDVNRASQKPSRRQLSNDNEL  517  DQ088990
N. scutatus     481  VHGWLDSTIKSGLTAARDVNRASQKPSRRQLSNDNEL  517  DQ088991
P. australis    481  VHGWLDSTIKSGLTAARDVNLASQKPSRIQLSNDNEL  517  DQ088992
A. blomhoffi    480  AHGWIDSTIKSGLRAARDVNRASEQ-----------  504  AB072393
C. rhodostoma   480  AHGWIDSTIKSGLRAARDVNLASENPSGIHLSNDNEL  516  AJ271725
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Figure 3.14. L-amino acid oxidase protein alignment.

Alignment of the deduced amino acid sequence from multiple cDNA clones of L-amino acid oxidases identified from Australian elapids compared to the previously characterized proteins from the Mamushi snake (*Gloydius blomhoffi*) and Malayan pit viper (*Calloselasma rhodostoma*). N-linked glycosylation sites previously identified in *C. rhodostoma* are also shown, as are the signal peptide and mature protein sequence. Genbank accession numbers are provided at the end of the sequences.

A full-length 1554bp LAAO coding sequence was amplified from the venom gland cDNA of *O. scutellatus*, *N. scutatus* and *P. australis*. Alignment of the deduced amino acid sequence from multiple cDNA clones reveal 89.6% identity across the three precursor proteins (figure

3.14). There was a high degree of similarity within the signal peptide, a common feature of all Australian elapid toxins identified by screens of the microarray. Previous investigations of L-amino acid oxidase activity from the crude venom of Australian elapids demonstrated *P. australis* to have relatively high levels of activity, *N. scutatus* to have moderate activity and very low activity rates in *O. scutellatus* (Tan and Ponnudurai, 1990). Given the significant degree of identity in the deduced primary structure of the LAAOs identified in this study, it would suggest the comparable rates of activity observed by Tan and Ponnudurai (1990) might be attributed to the relative quantity of LAAO in the venom. However, one notable difference between the Australian elapids sequences is the absence of a second N-linked glycosylation site in *P. australis* that has previously been observed in *Calloselasma rhodostoma*, although it is difficult to draw conclusions as to the effect on activity of this variation (MacHeroux *et al.*, 2001).

Discussion

Given the significant clinical effects of Australian elapid venoms, and their as yet relatively understudied nature at the molecular level, a cDNA microarray chip was established from the venom gland of the coastal taipan (*O. scutellatus*) for the purposes of cross-species comparisons and the identification of venom gland specific transcripts. By hybridising RNA isolated from the liver of *O. scutellatus* it was possible to select a suite of genes that demonstrated increased expression in the venom gland compared to the liver. It was hypothesised that these transcripts play a role specific to that tissue, for example coding for a toxin for secretion into the venom. Of the 58 unique transcripts identified from screens of the microarray chip, 34 demonstrated increased expression in the venom gland when compared to the liver. All clones whose putative function determined via BLAST homology searches indicated a role as a venom toxin, or a role in the processing and secretion of toxins into the venom, were amongst these clones which demonstrated an increase in expression in the venom gland. Hence, hybridising a venom gland microarray chip with liver RNA has proven a valuable method for the screening and identification of unique toxin genes within the entire transcriptome represented by a venom gland cDNA library. It was possible to further verify these results by screening the microarray chip with RNA isolated from the venom gland of the closely related taipan, *O. microlepidotus*, in association with quantitative PCR analysis, which confirmed that indeed none of these genes were expressed in the liver.

Of the known toxin genes identified the majority of clones represented on the chip corresponded to neurotoxins, phospholipase A_2 enzymes and a pseudechetoxin-like protein, along with a calglandulin-like protein that has previously been implicated in the export of toxins out of the venom gland (table 3.02). In fact, these four families of proteins represented 77% of the clones sequenced from the microarray chip. This high level of redundancy may be attributed to the fact that the RNA used for the formation of the cDNA library was from a single tissue source with a highly specific function, that is the production of venom, and hence the majority of clones within the transcriptome of this tissue will be represented by these toxin sequences. This, in addition to the fact that screening of the microarray was tailored towards venom gland specific genes, resulted in the high number of toxin/calglandulin-like genes sequenced. The predominance of venom gland specific genes on the microarray chip is further evidenced by the bias observed in the expression level of genes in the histogram of figure 3.02, which compares venom gland expression to liver expression.

The number of clones identified for each gene does not necessarily reflect their relative expression within the *O. scutellatus* venom gland, nor the amount of protein secreted into the venom, as bias towards certain clones will inevitably be introduced into the cDNA library through the process of its amplification and size fractionation. Similarly, it is difficult to draw conclusions about the relative expression of each toxin gene within the venom gland of any Australian elapid represented in table 3.03, as numerous isoforms exist for many of these genes, not to mention variation in sequence between species, which will affect hybridisation of transcripts to the chip. The one notable exception is the calglandulin-like transcript, which was present within all snakes as a single clone with significant identity between the species. Another noteworthy feature of table 3.03 is that *T. carinatus* had the lowest expression level of pseudechetoxin-like protein, whilst *P. textilis* has the lowest expression levels for the short chain neurotoxin arrayed on the chip, which is in agreement with subsequent findings that these products could not be cloned from these snakes.

A number of clones that demonstrated either increased or decreased expression in the venom gland of *O. scutellatus* represented regulatory genes involved in normal cellular maintenance. These genes included ribosomal proteins, creatine kinases, polyadenylate binding-protein homologs, and other regulatory proteins typically expected to be found in most cell types. One of these transcripts, a 60S ribosomal protein L13a homolog, showed no greater than an

average two-fold difference in expression level between all elapid venom glands and liver samples. This equivalent expression in all tissue made it an ideal candidate for a house-keeping gene for all quantitative PCR experiments performed.

Screens of the microarray also identified a total of 24 unique clones that demonstrated no known homology to other transcripts via a BLAST search, not surprising since there is little prior information on the protein and nucleotide sequences from Australian elapids. Eight of these clones demonstrated an increase in expression in the venom gland, and may represent as yet unique toxin sequences, and warrant further investigation via 5'-RACE and functional analysis. A completely novel toxin or toxin family, with unique biological functions represent attractive candidates for further investigation as a potential therapeutic or diagnostic agent. Interestingly, no clones identified by examination of the microarray chip returned matches for the factor X-like or factor V-like components previously shown to be present within the venom gland cDNA and venom of *O. scutellatus* in chapter 2. This may have resulted from one or more possibilities: the gene was sequenced from the array but could not be identified by BLAST searches as the library clone only represented 3'-UTR sequence which is not represented on the database; these clones are present in low abundance in the venom gland transcriptome or bias toward other transcripts (for example calglandulin-like protein) introduced during the cDNA amplification process resulted in the presence of this clone in low abundance in the library; or finally, it is possible that these clones are present as some of the 4,800 clones on the microarray chip however were not selected for sequencing as they showed equivalent expression in the liver due to the presence of a highly similar prothrombin activator produced by the snake for its own coagulation cascade.

Subsequent to the identification of full-length venom gland specific genes within *O. scutellatus*, it was possible to identify homologous proteins within other related Australian snakes given the high degree of identity in primary sequence between these species. The first of such transcripts was a pseudechetoxin-like protein. Pseudechetoxin, and its related homolog pseudecin, are peptidic toxins that target cyclic nucleotide-gated (CNG) ion channels isolated from the venom of the mulga (*P. australis*) and red-bellied black snake (*P. porphyriacus*) respectively (Brown *et al.*, 1999). Full-length cDNA's coding for both of these toxins have recently been reported and have been shown to bind the pore turret of CNG ion cannels and inhibit the flow of current (Brown *et al.*, 2003; Yamazaki *et al.*, 2002). CNG ion channels, which have been identified in a number of body tissues including the brain, heart

and kidneys, play a central role in signal transduction in retinal photoreceptors and olfactory neurons. They modulate the membrane potential of the cell and intracellular calcium levels in response to stimulus-induced changes in cyclic nucleotide concentration (Burns and Baylor, 2001; Bradley *et al.*, 1997).

Overall the pseudechetoxin-like proteins identified in this study exhibit a significant degree of identity (83%) across the entire precursor molecule. The *P. australis* and *P. porphyriacus* sequences identified in this study were identical to past findings by Yamazaki *et al.* (2002) (figure 3.04). However, even though only 7 amino acid differences were observed between the *P. australis* and *P. porphyriacus* mature protein sequences, a 30-fold difference in affinity for CNG ion channels was previously observed between the two. Comparison of the *P. australis* sequences with that of the *O. scutellatus* sequence reveals a 23 amino acid differences in the mature toxin sequence, suggesting that there may be large differences in capacity to bind to ion channels for the pseudechetoxin-like proteins from different elapids. Hence, the data described here represents a valuable tool for further probing the structure and function of CNG ion channels.

The phylogenetic relationship described for pseudechetoxin-like proteins (which is based on deduced amino acid sequence for a specific toxin gene) is in good agreement with the evolutionary relationship of elapid snakes which have previously been based on a range of other parameters including internal and external morphology, immunological distances, ecological and biochemical means (Hutchinson, 1990). Advances in DNA and protein sequencing technologies have seen the incorporation of molecular means for establishing phylogenetic relationships between snakes, that is, the inference of species trees from gene trees (Fry and Wuster, 2004; Slowinski *et al.*, 1997). Molecular sequence data may prove to be a powerful tool in that not only can it indicate the order of divergence between species but also give a measure of the timing of that divergence (Wuster *et al.*, 2005). The phylogenetic relationship described here for pseudechetoxin closely resembles that of the factor X-like protease family described in chapter 2.

Yamazaki *et al.* (2002) reported the processing of a 211 and 210 amino acid mature protein for pseudechetoxin and pseudecin respectively. This difference in size was due to the presence of an extra serine residue at the N-terminus of pseudechetoxin as determined by Edman degradation. This is of interest because the recognition sequence between the signal

peptide and mature protein is 100% conserved within these two species. This suggests that in the case of pseudechetoxin, a proteolytic cleavage occurs between the two serine residues (SES↓SNK) while for pseudecin the cleavage site is SESS↓NK. The data provided in figure 3.04 demonstrates that the signal peptide is highly conserved in all seven Australian elapids and the region surrounding the proposed cleavage site (SESSNK) is identical. This suggests that processing of the precursor protein occurs by cleavage at a single site and that the single amino acid difference reported in *P. porphyriacus* might be explained by cleavage of the N-terminal serine from pseudecin during the purification procedure.

Identification of venom gland specific transcripts from the *O. scutellatus* microarray was not limited to only toxins, indeed the most abundant clone identified (approximately 35% of all clones screened) corresponded to a calglandulin-like protein. Calglandulin is an EF handed protein with conserved Ca^{2+} motifs, first identified from the venom gland of the Island Jaracara, *Bothrops insularis* and has been implicated in the process of exporting toxins out of the cell and into the venom (Junqueira-de-Azevedo Ide *et al.*, 2003). Members of the EF handed protein family contain conserved Ca^{2+} binding motifs involved in intracellular communication, vesicular transport and membrane fusion, the most widely studied of these being calmodulin (Chin and Means, 2000; Niki *et al.*, 1996). Calglandulin displays significant structural homology to calmodulin, both consisting of four EF hands (the helix-loop-helix motif that coordinates Ca^{2+} binding) and hence has been implicated in the secretion of toxins from the venom gland cell and into the venom. This is supported by the localisation of the protein specifically to the venom gland. Given the significant identity observed between calglandulin and the homolog identified in Australian elapids in this study, in conjunction with their absence from the liver, it is suggested that these proteins play a similar role within the venom gland of Australian snakes (figure 3.08, figure 3.10). Such a high degree of conservation between two different families of snakes (vipers and elapids) may be indicative of the highly specific function of these proteins. Interestingly a novel EF hand protein with 80% identity to calglandulin has been isolated and cloned from the human skeletal muscle, CAGLP (Chen *et al.*, 2004). The homologs identified in Australian elapids, along with Calglandulin and human CAGLP, represent a distinct group within the EF handed Ca^{2+} binding protein family.

Phospholipase A_2 (PLA$_2$) enzymes are a ubiquitous family of proteins present within many venomous animals including Australian snakes. Of the three PLA$_2$ isoforms identified from

screens of the microarray chip, the most abundant corresponded to a full-length mRNA transcript of the β-chain of taipoxin (the others were only short segments of 3'-UTR with no coding sequence). Taipoxin is a potently active toxin within the venom of the coastal taipan and is composed of three PLA$_2$ chains: α, β and γ (Fohlman *et al.*, 1976). The neurotoxic and myotoxic effects of taipoxin can be attributed to the α-chain, with the presence of the γ-chain enhancing these effects (Harris and Maltin, 1982). Interestingly, the β-chain is neither toxic nor enzymatically active on its own, and somewhat surprisingly has been shown to have mitogenic activity (Lipps, 2000; Lind, 1982). This is despite 61% identity with α-taipoxin and the mature protein level. Three other PLA$_2$ enzymes have previously been identified at the protein level from the venom of *O. scutellatus* including secretory phospholipases OS-1 and OS-2, as well as a PLA$_2$ chain present within the multimeric protein taicatoxin, however no full-length cDNAs have previously been described from this snake (Lambeau *et al.*, 1995; Possani *et al.*, 1992b; Possani *et al.*, 1992a). This study represents the first description of the cloning of a PLA$_2$ from the venom gland of *O. scutellatus*.

Using primers designed from the cDNA clone of β-taipoxin, it was possible to amplify multiple PLA$_2$s not only from *O. scutellatus*, but also within most of the other Australian elapids involved in the study. These clones displayed a range of sequence variation, even within a single species. However there were areas of conservation, most notably the propeptide sequences, as well as sequences involved in Ca^{2+} binding and the site responsible for phospholipase activity (figure 3.13). However due to subtle variation between proteins particularly within the C-terminal region of the molecule, many of these PLA$_2$s are predicted to have different activities that may potentially be applied in drug discovery setting. A perfect example of the characterisation of a PLA$_2$ as a novel therapeutic is β-taipoxin (whose cDNA sequence is described for the first time here) which is being studied for its mitogenic/wound healing characteristics (Lipps, 2000). Similarly, meta-analysis of the phospholipase cDNAs described here in combination with the numerous other identified sequences from other hydrophids, vipers, crotalids and non-Australian elapids will provide further data on the evolutionary links between theses snakes.

In addition to being implicated in the digestion of prey, venom PLA$_2$s have been associated with a wide range of physiological functions including haemorrhagic, myotoxic, haemolytic, hypotensive, oedema forming, platelet aggregating, convulsant, cardiotoxic and pre- and post-

synaptic neurotoxic activities (Arni and Ward, 1996). This variation in activity is despite what appears to be a high degree of conservation in their primary structure, and may be independent of phospholipase catalytic activity. It is probable that the large number of PLA_2s described here from seven Australian elapids may also posses a variety of activities, however based on primary sequence alone it is difficult to assign specific activities to any one isoform. For example, despite displaying approximately 65% overall identity at the mature protein level between the venom PLA_2s scutoxin from *N. scutatus* and PA-9C from *P. australis*, one displays potent presynaptic neurotoxic effects, whilst the other demonstrates myotoxic activity, with variable phospholipase enzymatic activity (Fletcher and Jiang, 1995; Takasaki *et al.*, 1990). Similarly, while the α-chain of taipoxin is neurotoxic, the β-chain displays no toxicity or phospholipase activity (and indeed demonstrates mitogenic properties), despite being in complex with the α-chain in native venom and displaying 64% identity to each other at the mature protein level (Lipps, 2000; Lind, 1982; Lind and Eaker, 1982).

In total, 41 different PLA_2 isoforms from seven different snakes were identified, making this the most comprehensive study of venom phospholipases from Australian elapids to date. The abundance of PLA_2 variants described in this study represents not only a valuable tool for the investigation of the structural and functional relationship of these proteins, but also for the characterisation of novel clones as potential therapeutic or diagnostic candidates, particularly if demonstrated to have a unique function upon further analysis.

L-amino acid oxidases (LAAO) catalyse the oxidative deamination of L-amino acids, and are widely distributed in a number of organisms including their presence within snake venoms where they are postulated to be toxins (Du and Clemetson, 2002). A protein with L-amino acid oxidase activity has previously been isolated and partially characterised from the venom of *P. australis* (Stiles *et al.*, 1991). However, no protein or gene sequence has been identified from this snake, or from any other Australian elapid. Research focus on snake venom LAAO's has been primarily been directed at vipers (Stabeli *et al.*, 2004; MacHeroux *et al.*, 2001). Given the known LAAO activity from mulga venom, it was of interest to identify the cDNA coding for the *P. australis* peptide responsible for this activity and determine if this protein was present in other Australian elapids, even though it was not identified as a clone from the *O. scutellatus* microarray chip. A full-length cDNA was identified in three Australian snakes, *O. scutellatus*, *P. australis* and *N. scutatus*, although this does not mean that a homologous protein is absent from the venom gland of the other elapids. Again there

was a significant degree of identity in deduced protein sequence between these three species, making it difficult to identify structural reasons for the varied rates of activity reported for these proteins within native venom (Tan and Ponnudurai, 1990). The LAAO previously isolated from the venom of *P. australis,* while non-toxic and non-proteolytic, demonstrated potent antibacterial effects, possibly as a result of the catalytic release of hydrogen peroxide. It has been postulated that this antibacterial activity protects the snake from the putrification of prey during the digestion process (Stiles *et al.*, 1991). The native LAAO identified by Stiles *et al*. (1991) had a molecular weight of 56kDa, which corresponds well with the predicted molecular weight of 56.7kDa of the LAAO identified in this study. Given the well described antibacterial activity, as well as recently identified platelet aggregating and apoptotic effects of LAAO, these molecules are of significant pharmacological interest. This study represents the first cloning of a full-length L-amino acid oxidase transcript from the venom gland of an Australian elapid.

Despite demonstrating a relatively high degree of toxicity, Australian snake venoms have remained largely understudied at the molecular level, and relatively little is known about the clinical pathology of individual venom components. This is epitomised by the fact that just a handful of nucleotide and protein entries are present within the Genbank database for the inland taipan (*O. microlepidotus*) in spite of possessing one of the most potently toxic venoms known to mankind. To address this deficiency, a cDNA library was constructed from the venom gland of the closely related species *O. scutellatus* in order to identify genes coding for the pharmacologically active components present within venom. To this end, microarray methodology was employed as a means to identify and characterise genes specifically expressed in the venom gland. cDNA sequences obtained in this manner allowed the identification of homologous genes in related Australian elapids. This data will act as an important resource for the phylogenetic analysis and the study of the evolutionary history of all snake toxins. The present study of the *O. scutellatus* venom gland transcripts has identified a significant number of the components associated with venom toxicity, many of which represent their first description at the cDNA and/or protein level. This approach provides a useful means for the characterisation of multiple isoforms of a toxin family not readily distinguishable at the protein level (for example the phospholipases), as well as a method for the identification of completely novel venom gland transcripts.

CHAPTER 4

Identification and Characterisation of Short and Long Chain α-Neurotoxins From Australian Elapids

Neurotoxins present within Australian elapid snake venoms serve a primary role in the immobilisation of bitten prey via rapid and complete systemic paralysis. Ultimately this paralysis may result in death via asphyxiation. The toxins responsible for these effects are typically small, disulfide rich and specifically target and block with high affinity neuromuscular transmission via the postsynaptic skeletal (α1) acetylcholine receptor (Fry, 1999). The nicotinic acetylcholine receptor (nAChR) family are ligand-gated, cation-selective ion channels that span the cell membrane of nerves and muscle fibres. They contain various pentameric assemblies of structurally related subunits that consist of the agonist binding α subunits (α1-α9) and other subunits including β, γ, δ and ε subunits (Colquhoun and Patrick, 1997). Binding of the elapid postsynaptic neurotoxins does not result in ultrastructural changes to the cell and the paralytic effects are more easily reversed by antivenom than those of the presynaptic neurotoxins (Sutherland and Tibballs, 2001).

Australian snake α-neurotoxins are classified as either short or long based on the number of amino acids in the mature protein, although both display relatively similar function. Mature short chain α-neurotoxins are typically 60-62 amino acids in length with four disulfide bridges, whilst long chain α-neurotoxins have five internal disulfide bonds and are 66-79 (average 73) residues long (Tsetlin, 1999). The secretion of these proteins is aided by a 21 amino acid signal peptide in the precursor protein (Gong *et al.*, 1999). Interestingly, some toxins do not fall easily into these categories. Two neurotoxins, Lc a and b from the yellow-lipped sea krait (*Laticauda colubrina*), are long chain neurotoxins 69 amino acids in length but only have four disulfide bonds. These toxins however, still demonstrate lethality in mice (Kim and Tamiya, 1982). Other than variation in the kinetics of association/dissociation with AChRs, the only other major functional difference between short and long chain α-neurotoxins is that only long chain toxins potently block α7 homo-oligomeric neuronal AChRs (Servent *et al.*, 1997). Both groups share a basic three-finger loop structure, with the majority of variation at the C-terminus, and occur in a monomeric form (Tsetlin, 1999; Golovanov *et al.*, 1993).

The first amino acid sequence of a venom neurotoxin, Tx α from the spitting cobra (*Naja nigricollis*), was identified in 1967 (Eaker and Porath, 1967). Since then, hundereds of neurotoxin peptide sequences have been identified worldwide from various snakes, typically with a molecular weight of around 6kDa (Phui Yee *et al.*, 2004). Perhaps the best characterised of these is bungarotoxin from another elapid, the banded krait (*Bungarus fasciatus*), used extensively in the study of acetylcholine receptors (Grant and Chiappinelli, 1985). Neurotoxins bind to the nAChR in a strictly competitive manner with native cholinergic agonists and antagonists, thereby blocking the receptor without induction of ion channel opening, blocking neurotransmission (Grant *et al.*, 1997). Only the α subunit of the nAChR is capable of binding neurotoxins, however while long chain neurotoxins can bind with high affinity to muscular and α7-neuronal nAChRs, short chain neurotoxins only bind to muscular nAChRs (Antil-Delbeke *et al.*, 2000). It is postulated that the presence of the fifth disulfide bond in long chain neurotoxins facilitate their binding to neuronal receptors (Servent *et al.*, 1997). Neurotoxin amino acid residues involved in the binding to particular receptors have also been characterised via chemical and site-directed mutagenic means (Pillet *et al.*, 1993; Tu *et al.*, 1971).

Although there has been much research focus on the structure of snake neurotoxins, and in particular their relationship with the mammalian nAChR, the postsynaptic neurotoxins of Australian elapids have remained largely understudied, particularly at the molecular level. This is surprising given the significant neuropathology and toxicity of the venom of these snakes. The single exception is the common brown snake, *P. textilis*, from which seven short chain and one long chain (pseudonajatoxin b) cDNA sequences have been cloned and identified (Gong *et al.*, 2001; Gong *et al.*, 2000; Gong *et al.*, 1999). These short chain neurotoxin isoforms were highly related, 57-58 amino acid mature proteins that demonstrated lethality similar to that of the native form when expressed recombinantly. Gong *et al.* (1999) suggest that these short chain neurotoxins have undergone an accelerated rate of evolution, as is observed in the phospholipase A_2 family (Kini and Chan, 1999; Ogawa *et al.*, 1992). Indeed, the entire genomic sequences for five of these toxins have been identified, with three exons and two introns capable of alternate splicing, and have been shown to have undergone gene duplication and accelerated evolution from an ancestral gene (Gong *et al.*, 2000). Pseudonajatoxin b, a long chain neurotoxin from the venom of *P. textilis* has also been cloned

and both the recombinant and native forms shown to be highly toxic in mammalian systems, particularly when compared to the short chain neurotoxins.

Appart from the toxins identified from *P. textilis*, there has been little research into these components within the venom of the other seven snakes involved in this study. The protein sequences of two neurotoxins, toxins 1 and 2 have been identified from the venom of the coastal taipan, *O. scutellatus* and differ by only a single amino acid (Zamudio *et al.*, 1996). These native proteins did not bind to neuronal AChRs and only weakly to muscular AChRs. A single mature protein sequence for both a short (Pa-a) and a long (Pa-ID) chain neurotoxin have been identified from the venom of the mulga (*P. australis*) (Takasaki, 1989; Takasaki and Tamiya, 1985). Pa-a demonstrates significant homology in primary sequence to toxins 1 and 2 from *O. scutellatus* and has an intravenous LD50 value of 0.076mg/kg (Takasaki and Tamiya, 1985). Pa-ID is a 68 amino acid long chain neurotoxin that does not demonstrate lethality in a mammalian system (Takasaki, 1989). Finally, a single long chain neurotoxin protein sequence has been characterised from the venom of the tiger snake, *N. scutatus*, and shown to be 73 amino acids in length and have an LD50 value of 0.125mg/kg (Halpert *et al.*, 1979). Aside from these five protein sequences no other neurotoxins have been identified from the venom glands of the snakes in this study, nor have there been any gene or cDNA sequences identified from these elapids. Indeed, no neurotoxins have ever been cloned from the venom glands of *O. microlepidotus*, *P. porphyriacus*, *T. carinatus* and *H. stephensii*, despite evidence to suggest their presence at various quantities within the venom of these snakes (Ramasamy *et al.*, 2005; Bell *et al.*, 1998).

Snake neurotoxins have played a vital role in understanding the structure and function of the mammalian nervous system, in particular the isolation and characterisation of the nicotinic cholinergic receptor at the motor end plate. The use of neurotoxins have been implicated in the study and treatment of a plethora of diseases including Alzheimer's disease, viral infection, myasthenia gravis, chronic pain/analgesics and thrombotic disease, as well as their employment as a general research tool, as reviewed by Phui Yee *et al*. (2003). Hence, this study aims to identify those components within the venom of Australian elapid snakes responsible for the postsynaptic neurotoxic effects observed upon envenomation. It is anticipated that characterisation of these components may lead to the identification and development of novel compounds with therapeutic or diagnostic benefits, as well as provide further evidence on the mechanisms of action of Australian snake venoms.

Methods and Materials

RNA Isolation and cDNA Synthesis

RNA was isolated and cDNA synthesised from the venom glands of a total of 8 Australian elapids as previously described in chapter 2. These snakes included the coastal taipan (*Oxyuranus scutellatus*), inland taipan (*Oxyuranus microlepidotus*), common brown snake (*Pseudonaja textilis*), red-bellied black snake (*Pseudechis porphyriacus*), mulga (*Pseudechis australis*), mainland tiger snake (*Notechis scutatus*), rough-scaled snake (*Tropidechis carinatus*) and Stephen's banded snake (*Hoplocephalus stephensii*).

Identification of Short Chain α-Neurotoxins

A transcript demonstrating significant homology to other short chain α-neurotoxins was identified in screens of the *O. scutellatus* microarray chip, and subsequently found in other Australian elapids by 5'-RACE and PCR analysis. 5'-RACE was performed from *O. scutellatus* cDNA template with a single gene specific primer (5'-GGT CGT CGA TGG ATG AGA GCA AAA CTC-3') as previously described, as the library clone only corresponded to a short region of coding sequence. Two bands were observed when the RACE product was run on a 1% TAE agarose gel, and both were excised, cloned and sequenced. Upon identification of the entire α-neurotoxin coding sequence, similar neurotoxin transcripts were identified in related snakes by PCR.

The short chain α-neurotoxin coding region was amplified from Australian snake cDNAs with forward (5'-CGC AAG ATG AAA ACT CTG CTG C-3') and reverse (5'-GCC ACT CGT AGA GCT AAT TGT TG-3') primers designed from the *O. scutellatus* sequence. The PCR reaction mixture contained approximately 200ng of cDNA template from all 8 elapids, 1unit of AmpliTaq gold buffered in 10mM Tris-HCl pH 8.3, 50mM KCl, 1.75mM $MgCl_2$ and 200μM dNTPs with 25ρmol of each primer in a final volume of 25μL. The reaction was thermocycled at 95°C for 10min followed by 30 cycles of 95°C for 30sec, 61°C for 40sec and 72°C for 40sec with a final extension of 72°C for 7min. All PCR products were run on a 1% TAE agarose gel, purified, cloned and sequenced as previously described. Alignments from multiple cDNA clones for each snake were performed using BioEdit software. For

phylogenetic analyses, the predicted protein sequences were aligned using ClustalW and subjected to pairwise deletion analysis using MEGA 2.1 software via the Neighbour-Joining method (1000 bootstrap replicates) (Kumar *et al.*, 2001). The *Thiobacillus denitrificans* Aldolase sequence YP314378 was used as an outgroup and a number of non-Australian snakes included in the analysis for an in depth comparison of short chain neurotoxin phylogenetics.

Identification of Long Chain α-Neurotoxins

A myriad of other putative long (and short) chain neurotoxins were identified from the venom gland cDNA of all Australian snakes involved in the study by PCR amplification. Primers were designed to hybridise within the 5'-coding region and 3'-UTR of the previously published *P. textilis* long chain neurotoxin Pseudonajatoxin b (AF082982). A 25μL PCR reaction was performed with cDNA prepared from the venom glands of all snakes, amplifying with 1unit of AmpliTaq Gold buffered in 10mM Tris-HCl pH 8.3, 50mM KCl, 1.7mM $MgCl_2$ and 200μM dNTPs with 25ρmol of each of the forward (5'-ATG AAA ACT CTG CTG CTG ACC-3') and reverse (5'-GTC GAG ATG TCA AAG ACG CA-3') primers. The reaction mixture was then thermocycled at 95°C for 8min, followed by 30 cycles of 95°C for 20sec, 59°C for 20sec and 72°C for 45sec with a final extension of 72°C for 7min. A single PCR product from each snake approximately 377bp in size were run on, and excised from, a 1% TAE agarose gel, purified and cloned as previously described. Multiple clones from each elapid were then sequenced and alignments of the cDNA and deduced amino acid sequences performed.

Cloning of Neurotoxins into pTWIN1

Two neurotoxins identified by PCR and sequene analysis were selected on the basis of novelty for further characterisation via recombinant expression and functional analysis. These included the putative short chain α-neurotoxin Os SNTX-1 from *O. scutellatus* and the putative long chain α-neurotoxin Pt LNTX-1 from *P. textilis*. Hence, full-length cDNA clones corresponding to both of these proteins were subcloned into the pTWIN1 vector for transfection and recombinant expression (New England Biolabs, Beverly, Massachusetts). The pTWIN1 vector utilises the IMPACT-TWIN (Intein Mediated Purification with an

Affinity Chitin-binding Tag - Two Intein) system, which allows for the purification of expressed constructs by specific binding of the protein to a chitin matrix, followed by cleavage of the target protein from a self inducible N-terminal intein tag upon a temperature and pH shift (Evans *et al.*, 1999). The vector also contains an optional C-terminal tag whose intein-mediated cleavage occurs in the presence of a reducing agent, which was not used in this experiment. A construct corresponding to the mature protein sequence without propeptide was cloned with the inclusion of the two amino acid residues, glycine and arginine, at the N-terminus of both to aid in downstream cleavage from the intein tag.

Cloning into the pTWIN1 vector was performed with the introduction of redundant Sap I restriction sites at the 5' and 3' ends of the previously cloned neurotoxin transcripts via PCR. Stop codons were included to ensure incorporation of the N-terminal chitin/intein tag, but not the C-terminal tag. A 50μL reaction was prepared with approximately 50ng of neurotoxin in pGEM-T vector as template for both constructs, 2 units of AmpliTaq gold buffered in 10mM Tris-HCl pH 8.3, 50mM KCl and 1.5mM $MgCl_2$ with 200μM dNTPs and 50ρmol of each of the forward and reverse primer. Os SNTX-1 was amplified with forward (5'-GGT GGT TGC TCT TCC AAC GGT AGA ATG ACA TGT TAC AAC CAA CAG TC-3') and reverse (5'-GGT GGT TGC TCT TCC GCA CTA ATT GTT GCA TTT GTC TGT TCT-3') primers, whilst Pt LNTX-1 was amplified with forward (5'-GGT GGT TGC TCT TCC AAC GGT AGA TTG ATA TGC TAC CTG GAT TTT AGT G-3') and reverse (5'-GGT GGT TGC TCT TCC GCA TCA ATG AGG TTT CTG TTT CGG-3') primers. The reactions were thermocycled initially at 95°C for 8min, then 30°C cycles of 95°C for 20sec, 57°C for 20sec and 72°C for 40sec with a final extension of 72°C for 7min.

A 3μL aliquot of the PCR product was run on a 1% TAE agarose gel to confirm its amplification. The remaining PCR product was purified via phenol:chloroform extraction and ethanol precipitated as previously described and the pellet resuspended in 11μL of water. After quantitation, approximately 3μg of PCR product was digested with 3units of Sap I buffered in 2-mM Tris-acetate pH 7.9, 50mM potassium acetate, 10mM magnesium acetate, 1mM dithiothreitol plus 100ng BSA in a final volume of 20μL at 37°C for 4hours (New England Biolabs, Beverly, Massachusetts). The pTWIN1 vector was similarly digested. The total product for each was run on a 1% TAE agarose gel stained with ethidium bromide and the digested bands excised and purified with a QIAex II Gel extraction kit as previously

described. The quantity of the purified product was then measured at an absorbance of 260nm.

The digested and purified PCR products for both constructs were then cloned in frame into the digested pTWIN1 plasmid by use of their matching 5' and 3' overhanging sequences. Ligation was performed with T4 DNA Ligase in the presence of 30mM Tris-HCl pH 7.8, 10mM $MgCl_2$, 10mM DTT and 1mM ATP 5% PEG in a final volume of 20μL at 16°C overnight. Insert to vector ratio was approximately 2:1. The ligation mixture was ethanol precipitated the following morning and used to transform electrocompetent dH5α *E. coli* cells as previously described. Multiple clones were selected and purified with a QIAprep spin miniprep kit and the presence of inserts confirmed by PCR with forward (5'-ACT GGG ACT CCA TCG TTT CT-3') and reverse (5'-GGC ACG ATG TCG GCG ATG -3') primers specific to the vector. Sequencing was also performed with these primers to ensure cloning of the correct construct.

Recombinant Neurotoxin Expression and Purification

Plasmids containing recombinant neurotoxin constructs in pTWIN1 were transformed into a number of electrocompetent *E. coli* cell lines including BL21 and ER2566. Cells were grown from an overnight starter culture in the presence of 100μg/mL of ampicillin at 37°C until they reached an OD of approximately 0.5, whereupon expression was induced via the addition of 0.5mM IPTG, shaking at 15°C overnight. Cells were then harvested by centrifugation at 4,000g for 12min at 4°C and resuspended in 50mL of chilled buffer B1 (20mM Tris-HCl pH 8.5, 500mM NaCl and 1mM EDTA) plus 0.15% Tween-20 and 20μM PMSF, for every 1L of original culture. A sample of uninduced culture with no IPTG was also similarly prepared for cross-comparisons. Induced and uninduced cells were then sonicated at 4°C and the debris removed via centrifugation at 13,000g for 10min at 4°C. Approximately 4mL of chitin beads were prepared by multiple washes with buffer B1, and then added to the cell lysate for protein binding on a rotating wheel at 4°C for 1hour (New England Biolabs, Beverly, Massachusetts). Unbound lysate was removed and stored and the beads washed three times with buffer B1, followed by a quick wash with buffer B2 (20mM Tris-HCl pH 7.0, 100mM NaCl and 1mM EDTA) at 4°C. Recombinant protein was then cleaved from the chitin-bound intein-tag via a downward shift in pH (to pH 7.0) and an increase in temperature to 25°C in 2mL of buffer B2

overnight. Additional recombinant protein was eluted with further 2mL washes of buffer B2 at room temperature, and a sample of the chitin beads also stored for analysis of the proportion of uncleaved protein. Purified recombinant protein products, as well as samples of induced and uninduced cell lysate, washes and the chitin beads, were then analysed on a 15% SDS-PAGE stained with coomassie as previously described. The recombinant Os SNTX-1 and Pt LNTX-1 proteins were then quantitated via a Lowry assay and sent to Dr Harald Fisher at the University of Queensland for further functional analysis as described in the two experiments below.

Catecholamine Secretion From Bovine Adrenal Chromaffin Cells

Chromaffin cells were prepared from bovine adrenal glands and maintained in 96-well plates as previously described (Meunier *et al.*, 2002). Intact cells were washed briefly once with buffer A (145mM NaCl, 5mM KCl, 1.2mM Na_2HPO_4, 10mM glucose and 20mM HEPES-NaOH, pH 7.4) and incubated with the recombinant neurotoxin constructs Os SNTX-1 and Pt LNTX-1 for 20min in the presence of 2mM $CaCl_2$, followed by stimulation with 5μM nicotine for 20min. Aliquots of the supernatant were taken at the end of each experiment and cells were lysed with 1% (v/v) Triton X-100. Total catecholamine release was examined in a fluorimetric assay and the amount released in the presence of the neurotoxins expressed as a percentage of the control.

Electrophysiological Recordings From Bovine Adrenal Chromaffin Cells

Chromaffin cells were prepared from bovine adrenal glands and maintained on glass cover slips in 24-well plates as previously described (Meunier *et al.*, 2002). Electrodes (GF150F-7.5, Harvard Apparatus Ltd., Edenbridge, UK) were pulled, fire-polished and resistance validated to be 2-3MΩ when filled with intracellular solution (140mM CsCl, 2mM $CaCl_2$, 11mM EDTA, 2mM MgATP and 10mM HEPES-KOH, pH 7.2). Agonists were diluted in bath solution (140mM NaCl, 3mM KCl, 1.2mM $MgCl_2$, 2.5mM $CaCl_2$, 7.7mM glucose and 10mM HEPES-NaOH, pH 7.35) and applied to cells by brief (10msec) pressure ejection (15psi, Picospritzer II, General Vale, Fairfield, NJ) from an extracellular patch pipette positioned 50-100μm from the cell soma to evoke maximal responses to agonists (Hogg *et al.*, 1999). Recombinant neurotoxins were then bath applied at varying concentrations (0.3 to

250µg/mL). Membrane currents evoked by agonist application were amplified and low-pass filtered (10kHz) using a MultiClamp 700B patch-clamp amplifier and voltage steps were generated using pCLAMP version 9.2 software and a Digidata 1322A interface (Axon Instruments Inc., Union City, CA). All experiments were carried out at room temperature.

Statistical analysis was performed with SigmaPlot version 8.0. All data represent the arithmetic means ± S.E.M. Full concentration-response curves for agonists were fitted by unweighted non-linear regression to the logistic equation:

$$E_x = E_{max}x^p/(x^p + EC_{50}{}^p), (1)$$

where E_x is the response, x the arithmetic dose, E_{max} the maximal response, p a slope factor and EC_{50} the dose that gives the half-maximal response. Curve fits to the logistic equation were carried out with the intention of providing estimates for EC_{50} values of agonists and for curve shifts caused by competitive antagonists. IC_{50} values were defined as the concentration of an antagonist that caused 50% inhibition of the response to a fixed concentration of an agonist.

Results

Identification of Short Chain α-Neurotoxins

A partial cDNA transcript for a short chain α-neurotoxin was identified from screens of the *O. scutellatus* microarray chip as previously described in chapter 3. Subsequent 5'-RACE identified a full-length transcript with a 252bp coding sequence from the venom gland of *O. scutellatus*. A BLAST search revealed significant homology at the nucleotide level to a short chain α-neurotoxin recently characterised from the venom of the marbled sea snake (*Aipysurus eydouxii*) as well as from the Hardwick's sea snake (*Lapemis hardwickii*) (Li *et al.*, 2005). This transcript coded for a protein 83 amino acids in length and was designated the name Os SNTX-1. The mature protein sequence for this neurotoxin, excluding propeptide, has previously been determined by Zamudio *et al*. (1996), who described the isolation and characterisation of two toxins from the venom of the coastal taipan which differed by only a single residue. Subsequently, primers were designed from the *O.*

scutellatus sequence to identify the presence of a similar product in the venom gland cDNA of related Australian elapids via PCR. A 271bp product, which included the full-length coding sequence, was obtained for all species examined in the study, and subsequently cloned and sequenced (figure 4.01A).
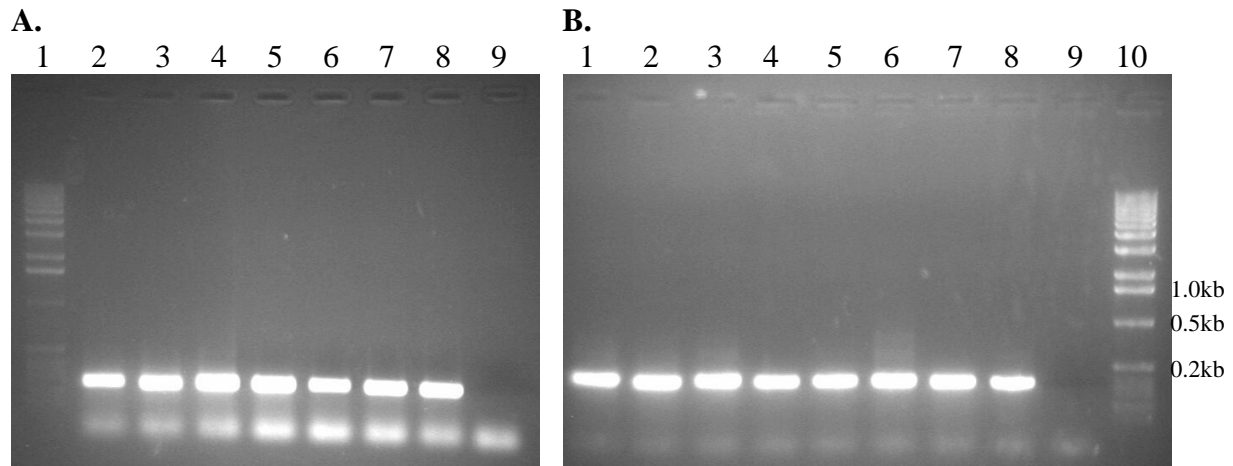

Figure 4.01. Full-length neurotoxin PCR amplification.

Amplification of **(A)** a short chain α-neurotoxin from venom gland cDNA isolated from 1) molecular weight marker, 2) *O. scutellatus*, 3) *P. textilis*, 4) *O. microlepidotus*, 5) *P. porphyriacus*, 6) *P. australis*, 7) *T. carinatus*, 8) *N. scutatus* and 9) negative control (a similar product was amplified from *H. stephensii* at a later date) and **(B)** a long chain α-neurotoxin from venom gland cDNA isolated from 1) *O. scutellatus*, 2) *P. textilis*, 3) *O. microlepidotus*, 4) *P. porphyriacus*, 5) *P. australis*, 6) *T. carinatus*, 7) *N. scutatus*, 8) *H. stephensii*, 9) negative control and 10) molecular weight marker.

PCR analysis using primers designed from Os SNTX-1 identified not only this toxin from the venom gland cDNA of *O. scutellatus*, but an additional putative short chain α-neurotoxin from the coastal taipan (designated Os SNTX-3 as a second toxin has previously been described by Zamudio *et al*. (1996)) (figure 4.02). BLAST searches indicate that Os SNTX-3 is a novel transcript, with closest homology at both the protein and nucleotide level to SNTX-6 from *P. textilis* (AF082980), however these two toxins only share 53% identity at the mature protein level (Gong *et al.*, 1999). When the PCR products were cloned and sequenced from the remaining seven snake species, a homologous short chain neurotoxin was identified in all but *P. textilis*. Again these proteins were assigned the name SNTX-1, with the exception of *T. carinatus* in which two clones were identified, differing by a single amino acid (N→H at position 51 of the precursor protein sequence). The novel clones identified

from *O. microlepidotus*, *P. porphyriacus*, *T. carinatus*, *H. stephensii* and *N. scutatus* represent the first description of a short chain α-neurotoxin at either the nucleotide or protein level from the venom gland of these snakes. The mature protein sequence of the toxin Pa-a has previously been reported from the venom of *P. australis* (P25497) and is 100% identical to the deduced sequence from the clone identified in this study (Takasaki and Tamiya, 1985). This toxin has been shown to produce peripheral paralysis by blocking neuromuscular transmission at the post-synaptic site and has an intravenous LD50 value of 0.076mg/kg. Note that a PCR product was also amplified from the venom gland cDNA of *P. textilis*, however subsequent cloning and sequencing revealed a neurotoxin with little identity to the family of proteins identified from the other snakes (Pt SNTX-8) (figure 4.02). Of the eight snakes examined, *P. textilis* has previously been the subject of the greatest research with respect to the neurotoxic components of the venom at the molecular level (Gong *et al.*, 2000; Gong *et al.*, 1999). A total of seven short chain neurotoxin isoforms have been cloned from the venom gland of *P. textilis*, and the transcript described in this study represents an eighth clone which has closest homology to that of Pt SNTX-1.

Figure 4.02 represents an alignment of the deduced amino acid sequence of all short chain α-neurotoxins identified from the venom glands of eight Australian elapids compared to five of the closest previously characterised toxins. The propeptide sequence is highly homologous between all proteins from different species, as well as within the species in the case of Os SNTX-1 and Os SNTX-3 from *O. scutellatus*, which in part may account for why the same primer pair detected two different products from the same sample of cDNA. This 21 amino acid sequence contains approximately 62% hydrophobic residues, consistent with its role as a secretion signal peptide. The cysteine residues involved in the formation of the four disulfide bonds that are a common feature of all short chain neurotoxins are 100% conserved amongst all Australian elapid species. Phylogenetic analysis of the SNTX-1 sequences identified in this study along with the other snake neurotoxins detailed in figure 4.02, show a distinct clustering of the Australian elapid sequences, with the *N. sputarix* toxin demonstrating significant homology to the *Oxyuranus* species, whilst the sea snake sequences also tend to cluster together (figure 4.03). As it was for the other phylogenetic trees reported in this thesis, significant identity between the two *Pseudechis* species as well as clustering of *N. scutatus*, *T. carinatus* and *H. stephensii* is again evident.
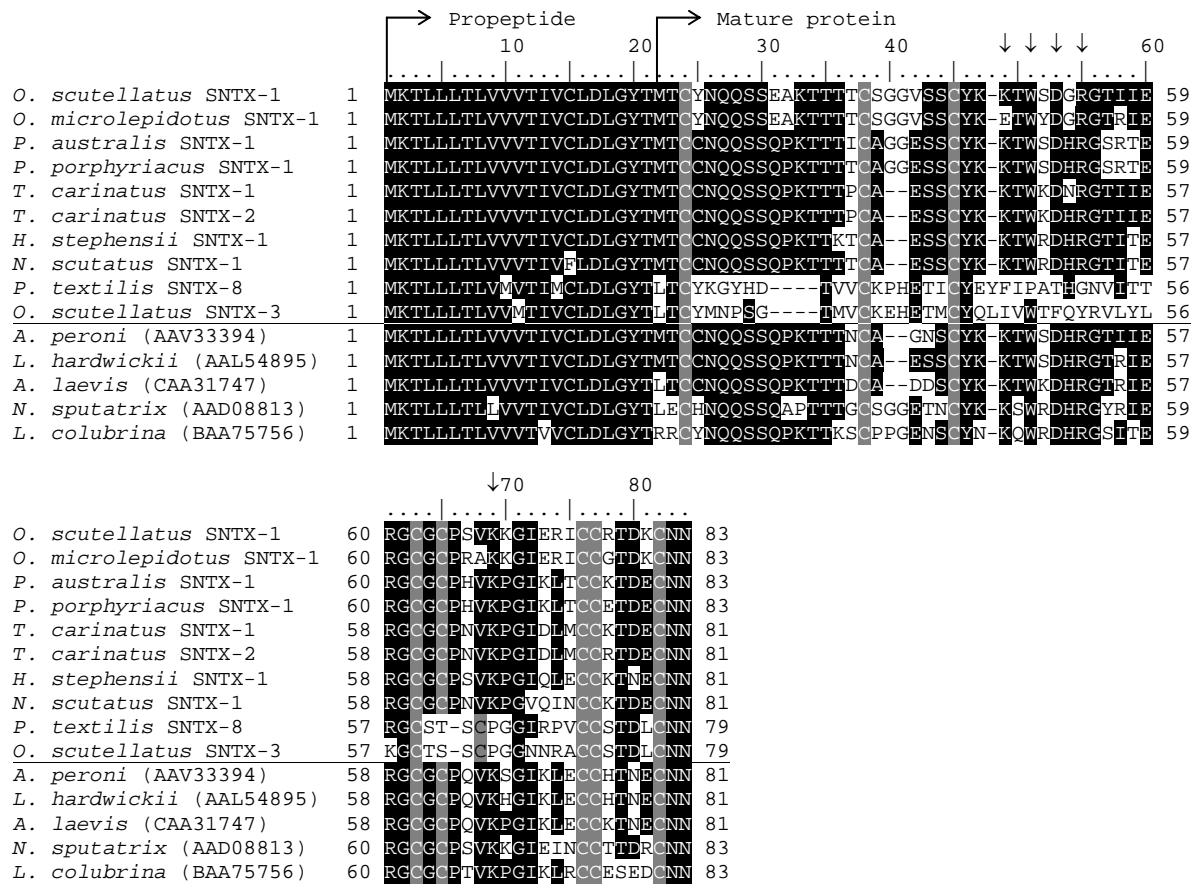
```
                              Propeptide          Mature protein
                              →                   →
                                   10        20        30        40           60
                              ....|....|....|....|....|....|....|....|   ↓ ↓ ↓ ↓  |
O. scutellatus SNTX-1     1   MKTLLLTLVVVTIVCLDLGYTMTCYNQQSSEAKTTTTCSGGVSSCYK-KTWSDGRGTIIE   59
O. microlepidotus SNTX-1  1   MKTLLLTLVVVTIVCLDLGYTMTCYNQQSSEAKTTTTCSGGVSSCYK-ETWYDGRGTRIE   59
P. australis SNTX-1       1   MKTLLLTLVVVTIVCLDLGYTMTCCNQQSSQPKTTTICAGGESSCYK-KTWSDHRGSRTE   59
P. porphyriacus SNTX-1    1   MKTLLLTLVVVTIVCLDLGYTMTCCNQQSSQPKTTTTCAGGESSCYK-KTWSDHRGSRTE   59
T. carinatus SNTX-1       1   MKTLLLTLVVVTIVCLDLGYTMTCCNQQSSQPKTTTPCA--ESSCYK-KTWKDNRGTIIE   57
T. carinatus SNTX-2       1   MKTLLLTLVVVTIVCLDLGYTMTCCNQQSSQPKTTTPCA--ESSCYK-KTWKDHRGTIIE   57
H. stephensii SNTX-1      1   MKTLLLTLVVVTIVCLDLGYTMTCCNQQSSQPKTTKTCA--ESSCYK-KTWRDHRGTITE   57
N. scutatus SNTX-1        1   MKTLLLTLVVVTIVFLDLGYTMTCCNQQSSQPKTTTTCA--ESSCYK-KTWRDHRGTITE   57
P. textilis SNTX-8        1   MKTLLLTLVMVTIMCLDLGYTLTCYKGYHD----IVVCKPHETICYEYFIPATEGNVITT   56
O. scutellatus SNTX-3     1   MKTLLLTLVVMTIVCLDLGYTLTCYMNPSG----TMVCKEHETMCYQLIVWTFQYRVLYL   56
A. peroni (AAV33394)      1   MKTLLLTLVVVTIVCLDLGYTMTCCNQQSSQPKTTTNCA--GNSCYK-KTWSDHRGTIIE   57
L. hardwickii (AAL54895)  1   MKTLLLTLVVVTIVCLDLGYTMTCCNQQSSQPKTTTNCA--ESSCYK-KTWSDHRGTRIE   57
A. laevis (CAA31747)      1   MKTLLLTLVVVTIVCLDLGYTLTCCNQQSSQPKTTTDCA--DDSCYK-KTWKDHRGTRIE   57
N. sputatrix (AAD08813)   1   MKTLLLTLLVVTIVCLDLGYTLECHNQQSSQAPTTTGCSGGETNCYK-KSWRDHRGYRIE   59
L. colubrina (BAA75756)   1   MKTLLLTLVVVTVVCLDLGYTRRCYNQQSSQPKTTKSCPPGENSCYN-KQWRDHRGSITE   59

                              ↓70          80
                              ....|....|....|....|....
O. scutellatus SNTX-1    60   RGCGCPSVKKGIERICCRTDKCNN   83
O. microlepidotus SNTX-1 60   RGCGCPRAKKGIERICCGTDKCNN   83
P. australis SNTX-1      60   RGCGCPHVKPGIKLTCCKTDECNN   83
P. porphyriacus SNTX-1   60   RGCGCPHVKPGIKLTCCETDECNN   83
T. carinatus SNTX-1      58   RGCGCPNVKPGIDLMCCKTDECNN   81
T. carinatus SNTX-2      58   RGCGCPNVKPGIDLMCCRTDECNN   81
H. stephensii SNTX-1     58   RGCGCPSVKPGIQLECCKTNECNN   81
N. scutatus SNTX-1       58   RGCGCPNVKPGVQINCCKTDECNN   81
P. textilis SNTX-8       57   RGCST-SCPGGIRPVCCSTDLCNN   79
O. scutellatus SNTX-3    57   KGCTS-SCPGGNNRACCSTDLCNN   79
A. peroni (AAV33394)     58   RGCGCPQVKGIKLECCHTNECNN    81
L. hardwickii (AAL54895) 58   RGCGCPQVKHGIKLECCHTNECNN   81
A. laevis (CAA31747)     58   RGCGCPQVKPGIKLECCKTNECNN   81
N. sputatrix (AAD08813)  60   RGCGCPSVKKGIEINCCTTDRCNN   83
L. colubrina (BAA75756)  60   RGCGCPTVKPGIKLRCCESEDCNN   83
```

Figure 4.02. Protein alignment of short chain α-neurotoxins.

Comparison is made between sequences identified in eight Australian elapid snakes (above the line) compared to the previously characterised toxins from the horned sea snake (*Acalytophis peroni*), Hardwick's sea snake (*Lapemis hardwickii*), olive sea snake (*Aipysurus laevis*), spitting cobra (*Naja sputatrix*) and yellow-lipped sea snake (*Lacticauda colubrine*). Conserved cysteine residues involved in putative disulfide bond formation are shaded gray, and propeptide and mature protein sequences denoted by arrows. Genbank accession numbers for formerly identified sequences are in brackets and the nomenclature previously reported for *P. textilis* and *O. scutellatus* is followed. Conserved residues implicated in receptor binding are denoted by ↓.
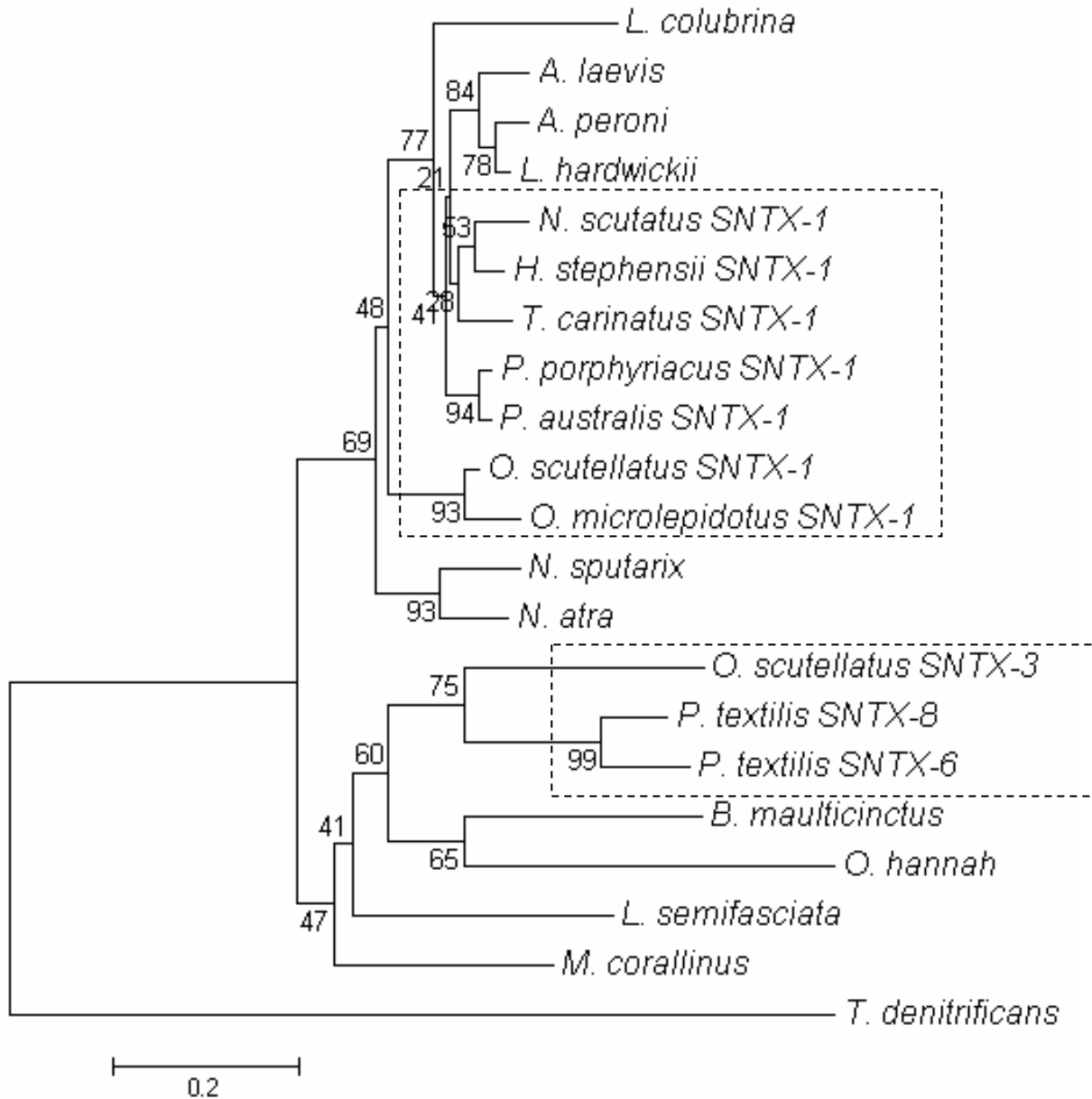
Figure 4.03. Phylogenetic relationship of short chain α-neurotoxin proteins.
Phyologenetic relationship of short chain neurotoxins as determined by the neighbour-joining method. Australian terrestrial snake sequences are indicated by a dotted box. The non-toxin *Thiobacillus denitrificans* sequence YP314378 was defined as an outgroup for the analysis. Other non-Australian toxin sequences include those depicted in figure 4.02 as well as neurotoxins from *Naja atra* (AAB86636), *Bungarus maulticinctus* (CAA45882), *Ohhiophagus hannah* (AAT97256), *Laticauda semifasciata* (BAC78204) and *Micrurus corallinus* (CAC50565)

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Identification of Long Chain α-Neurotoxins

A long chain α-neurotoxin, pseudonajatoxin b, has previously been cloned and identified from the venom gland of the common brown snake *P. textilis* (Gong *et al.*, 2001). To further identify the presence of other long chain neurotoxins from related Australian elapids, primers were designed from the mRNA sequence of pseudonajatoxin b within the 5' and 3'-UTR regions of this gene. A single PCR product was amplified using these primers and subsequently cloned from all eight snakes (figure 4.01B). An alignment of the deduced amino acid sequence of all unique long chain α-neurotoxins identified is shown in figure 4.04, compared to that of the published pseudonajatoxin b sequence. These transcripts have been defined as long chain neurotoxins on the basis of the length of their mature protein sequences (68 to 73 amino acids) and homology as a result of BLAST searches. Interestingly, although primers were designed specifically for the pseudonajatoxin b sequence, this transcript was not identified in the PCR from *P. textilis* cDNA. A second toxin was identified however, called Pt LNTX-1, which showed little structural homology to pseudonajatoxin b. Indeed the closest known match to the mature protein sequence is a long chain neurotoxin from the yellow-lipped sea krait (*Laticauda colubrina*), neurotoxin b (0901189B). This toxin demonstrates only 51.4% identity between the mature toxins, indicating that Pt LNTX-1 represents a novel neurotoxin. Interestingly, although mature Pt LNTX-1 is 72 amino acids in length, it does not contain the conserved cysteine residues that form the fifth disulfide bond characteristic of long chain neurotoxins, and has an alternate cleavage site between the signal peptide and mature protein (figure 4.04).

The putative long chain neurotoxins identified in the other Australian elapids varied depending on which snake they were derived from. Os LNTX-1 from *O. scutellatus* and Om LNTX-1 and LNTX-2 from *O. microlepidotus* are highly related to each other (90.2%) and to pseudonajatoxin b from *P. textilis*, however they lack the 11 amino acid C-terminal tail present within pseudonajatoxin b. One notable difference however is the absence of the cysteine residues involved in the formation of the fifth disulfide bond in the *O. scutellatus* sequence, which is a discriminating feature between long and short chain neurotoxins. These sequences represent the first description of a long chain neurotoxin from the venom of an *Oxyuranus* species. Interestingly, the same PCR product from *O. scutellatus* also contained Os SNTX-1 clones described in figure 4.02.

```
α-cobratoxin                                                     K  WCDAFC   R  KR
                          Propeptide          Mature protein      ↓ ↓ ↓     ↓  ↓
                          10        20        30        40        50        60
                          ...|....|....|....|....|....|....|....|....|....|....|
N. scutatus LNTX-1      1  MKTLLLTLVVVTIVCLDLGDSLICYMGPKTP-RTCPRGQNLCYTKTWCDAFCSSRGKVVE  59
T. carinatus LNTX-1     1  MKTLLLTLVVVTIVCLDLGNSFSCYKTPHVKSEPCAPGQNLCYTKTWCDAFCFSRGRVIE  60
P. australis LNTX-ID    1  MKTLLLTLVVVTIMCLDLGYTLTCYKGRDRSSETCRSEQELCCTKTWCDQWCQDRGPRLE  60
P. textilis LNTX-1      1  MKTLLLTLVVVTIVCLDLGDSLICYLDFSVP-HTCAPGEKLCYTRTWND----GRGTRIE  55
O. microlepidotus LNTX-1 1 MKTLLLTLVVVTIVCLDLGYTRRCFITPDVRSERCPPGQEVCYTKTWCDGFCGSRGKRVD  60
O. microlepidotus LNTX-2 1 MKTLLLTLVVVTIVCLDLGYTRRCFITPDVRSERCPPGQEVCYTKTWCDGFCSSRGKRVD  60
O. scutellatus LNTX-1   1  MKTLLLTLVVVTIVCLDLGYTRRCFTTPSVRSERCPPGQEVCYTKTWTDGHGGSRGKRVD  60
Pseudonajatoxin b       1  MKTLLLTLVVVTIVCLDLGYTRTCFITPDVKSKPCPPGQEVCYTKTWCDGFCGIRGKRVD  60


α-cobratoxin                         K                 F
                                     70        80      ↓  90        100
                                     ...|....|....|....|....|....|....|....|...
N. scutatus LNTX-1      60  LGCAATCPIAKSYE-DVTCCSTDNCNPFPVRPRPHP-------  94
T. carinatus LNTX-1     61  LGCAATCPPAEPKK-DISCCSTDNCNPHPAHQSR---------  93
P. australis LNTX-ID    61  MGCTATCPR-RMPGLDFTCCTTDNCNPVPT------------  89
P. textilis LNTX-1      56  RGCAATCPIPKKPEIHVTCCSTDRCNPHPKQ-KPH--------  89
O. microlepidotus LNTX-1 61 LGCAATCPTPKKKDIKIICCSKDNCNTFPKWP----------  92
O. microlepidotus LNTX-2 61 LGCAATCPTPKKKGIDIICCSKDNCNTFPKWP----------  92
O. scutellatus LNTX-1   61  LGCAATCPTPKKKDIKIICCSTDNCNTFPKWP----------  92
Pseudonajatoxin b       61  LGCAATCPTPKKTGIDIICCSTDDCNTFPLRPRGRLSSIKDHP 103
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Figure 4.04. Protein alignment of long chain α-neurotoxins.

Sequences identified in Australian elapid snakes are compared to pseudonajatoxin b (AAK15774) previously characterised from the venom of *P. textilis* (Gong *et al.*, 2001). Propeptide and mature protein sequences are shown and conserved cysteine residues involved in putative disulfide bond formation shaded gray. Residues implicated in the binding of both muscular and neuronal AChRs by α-cobratoxin are denoted by ↓ (Antil *et al.*, 1999). Other residues which are required for either specifically binding just muscular AChRs or neuronal AChRs are also shown (the lysines shaded black are required for muscular AChR binding) (Antil-Delbeke *et al.*, 2000).

Other unique long chain neurotoxins were also identified from the venom gland of *N. scutatus* and *P. australis*, which have previously been described at the protein level, but not at the level of cDNA. PA-ID (P14612) is a long chain neurotoxin from the venom of *P. australis* previously identified by Takasaki (1989) and was shown to be non-lethal as it is unable to bind to the acetylcholine receptor. The deduced amino acid sequence of the clone identified in this study is 100% identical to that of PA-ID, and represents the first description of not only the mRNA and propeptide sequence for this protein, but of any nucleotide sequence of a long chain neurotoxin from this snake. PCR with the pseudonajatoxin b primers also identified Pa-a (figure 4.02) along with PA-ID. Only a single neurotoxin protein sequence (P01384) has been previously identified from the venom of *N. scutatus* (Halpert *et al.*, 1979). The mRNA transcript Ns LNTX-1 identified by PCR in this study codes for a 73 amino acid protein

similar to that described by Halpert *et al*. (1979), however the clone identified in this study differs by a reversal of the second last and third last amino acids compared to the published protien. It is possible that this is due to an error in the protein sequencing of the mature toxin, as these residues were present in that order in the multiple clones sequenced in this study, or these clones just represent a second isoform. The mature protein for this long chain neurotoxin has been shown to have an intravenous LD50 value of 0.125mg/kg in mice.

Finally a unique long chain neurotoxin was also identified in the venom gland of *T. carinatus*, Tc LNTX-1 (figure 4.04). This clone demonstrated little homology to the neurotoxins identified by PCR with primers designed from pseudonajatoxin b. However, BLAST searches reveal a homologous protein, neurotoxin-I (P01382), from *Naja oxiana* (Central Asian cobra) whose mature protein sequence demonstrated 69.9% identity to Tc LNTX-1, and has been shown to bind acetylcholine receptors and block neuromuscular transmission (Grishin *et al.*, 1974). It should be noted that a PCR product was also obtained from *P. porphyriacus* and *H. stephensii* (figure 4.01B) however subsequent cloning and sequencing revealed that the only transcript amplified were the short chain neurotoxins, Pp SNTX-1 and Hs SNTX-1, previously reported in figure 4.02. The signal peptide had a high degree of identity for all long chain neurotoxins, and also between long and short chain neurotoxins, suggesting a similar mechanism of secretion and activation in Australian elapids. This high degree of similarity may also suggest why different primer pairs for amplifying either short or long chain neurotoxins detected the same transcript in many instances.

Detection of Proteins in Whole Venom

The neurotoxic effects of Australian elapid envenomations have been well documented, however the components responsible for these clinical symptoms have remained poorly characterised at the molecular level. The short and long chain neurotoxins described for the eight snakes in this study in many cases represent the first description of a specific neurotoxin mRNA transcript for many of these snakes. To correlate these sequences identified in the transcriptome of each snake with actual secreted protein, whole venom was run on a 15% SDS-PAGE and stained with coomassie to detect the presence of low molecular weight proteins (figure 4.05). The theoretical molecular weight of the neurotoxins identified in this study were calculated to be in the range of 6.34kDa to 8.98kDa. These theoretical masses correlate well with the presence of multiple protein bands at this low molecular weight range

within the venom of the eight elapids evident in figure 4.05, although not all these bands may necessarily be neurotoxins. This result further supports the presence of numerous isoforms of both long and short chain α-neurotoxins within the venom of Australian snakes.
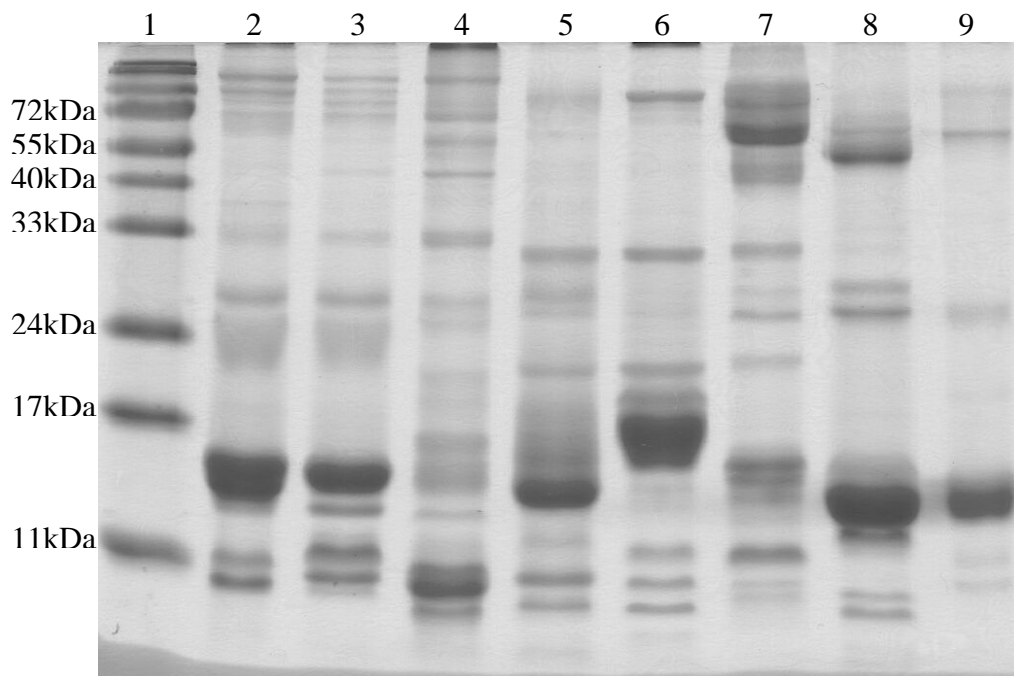
Figure 4.05. Australian elapid whole venoms.

Venoms were run on a 15% SDS-PAGE under reducing conditions and stained with colloidal coomassie. Lanes were loaded with whole venom from the following snakes as follows: 1) Pre-stained molecular weight marker, 2) *O. scutellatus*, 3) *O. microlepidotus*, 4) *P. textilis*, 5) *N. scutatus*, 6) *T. carinatus*, 7) *H. stephensii*, 8) *P. porphyriacus* and 9) *P. australis.* Note the presence of lower molecular weight peptides in all species of snake.

Recombinant Expression and Functional Determination of Neurotoxins

Two neurotoxins identified above were selected for further characterisation via recombinant protein expression and functional analysis. The IMPACT-TWIN *E. coli* protein expression system was selected due to a number of advantages it presented including the one step purification of large quantities of protein, which do not contain any tags, in a non-reducing environment (Xu and Evans, 2001). Numerous examples exist of the expression of functionally active neurotoxins from snakes and other organisms within an *E. coli* expression system (Peng *et al.*, 2003; Wang *et al.*, 2002; Kadkhodayan *et al.*, 2000; Gong *et al.*, 1999). The first toxin selected was Os SNTX-1 from *O. scutellatus*, a short chain α-neurotoxin that

has previously been characterised as a native protein from the venom of this elapid, and hence will form a suitable reference protein for comparisons performed with this expression system (Zamudio *et al.*, 1996). A homolog of this toxin was also identified in all other snakes and therefore is worthy of further characterisation. The second toxin selected was Pt LNTX-1 identified in *P. textilis* and was chosen on the basis that it represents a unique and novel long chain neurotoxin, not only from elapids but for any snake family.
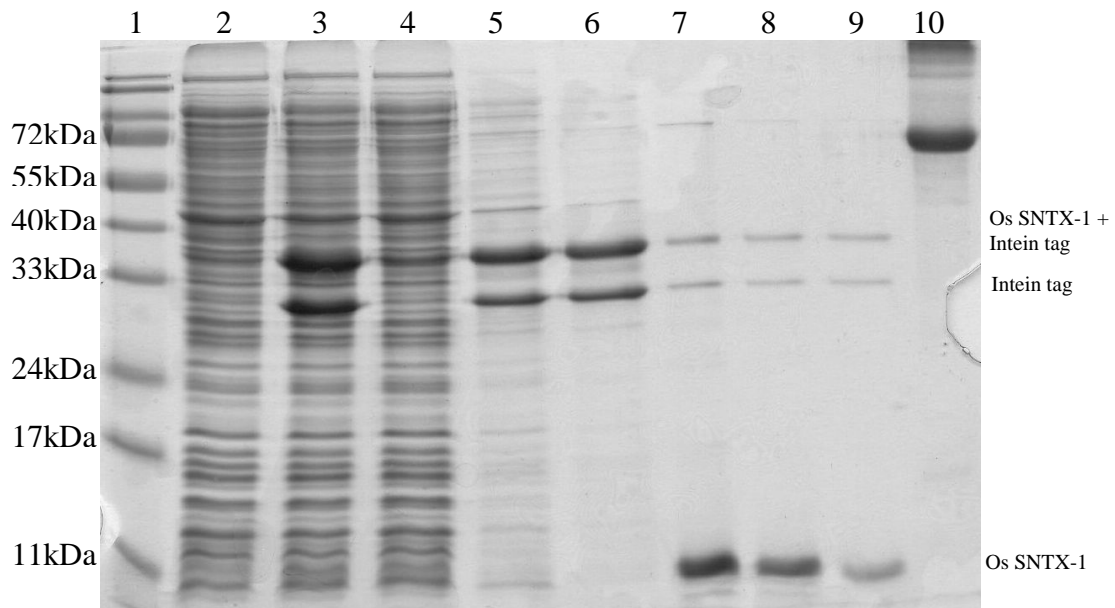
Figure 4.06. Purification of recombinant *O. scutellatus* SNTX-1.

Fractions were loaded on a 15% SDS-PAGE as follow: 1) Molecular weight marker, 2) Uninduced cell lysate, 3) Induced cell lysate, 4) Unbound cell lysate, 5) Column wash 1, 6) Column wash 4, 7) Elution 1, 8) Elution 2, 9) Elution 3 and 10) 5μg BSA. Note the presence of the cleaved recombinant protein in the elutions, along with residual chitin/intein tag.

Constructs for both neurotoxins were cloned into pTWIN1 and subsequently expressed and purified. A coomassie stained gel demonstrating the expressed Os SNTX-1 product is evident in figure 4.06. Note the presence of residual intein-tag bound protein within the final eluted sample, however the recombinant protein is of otherwise high purity. Protein concentration for both samples was determined via Lowry assay. Os SNTX-1 was recovered at a concentration of approximately 500μg/mL, whilst Pt LNTX-1 was only approximately 25μg/mL due to a decrease in the efficiency of cleavage of the protein from the tag. Both purified recombinant protein constructs were then sent to the University of Queensland for further functional characterisation.

Two assays were performed to gauge the interactions of both recombinant neurotoxins with nicotinic acetylcholine receptors. The first of these examined the potential for the toxin to inhibit the release of catecholamines/neurotransmitters from bovine chromaffin cells upon nicotine stimulation. The results of figure 4.07 indicate that whilst *O. scutellatus* SNTX-1 only demonstrated partial inhibition at high concentrations, the novel *P. textilis* LNTX-1 toxin was capable of significant inhibition of catecholamine release in a dose dependent manner. This assay, while indicative of the ability for the toxin to inhibit neurotransmission does not identify the point at which this inhibition occurs. To further scrutinize this relationship, electrophysiological recordings from bovine chromaffin cells in the presence of varying concentrations of each toxin were also examined.
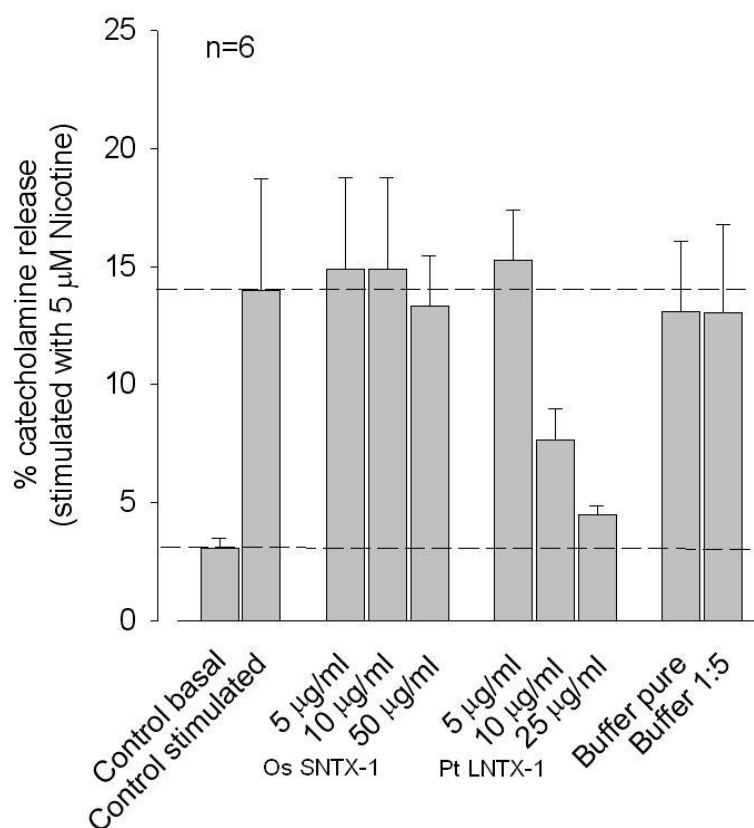
Figure 4.07. Graph of the inhibition of nicotine evoked catecholamine release from isolated bovine chromaffin cells.

The effect of increasing concentrations of OS SNTX-1 and Pt LNTX-1 recombinant neurotoxins on the basal level of catecholamine release is evident. Results using the experimental buffer were also included as a negative control. A greater dose dependent inhibition of catecholamine release is evident in Pt LNTX-1 than Os SNTX-1. Elution buffer is included as a negative control.
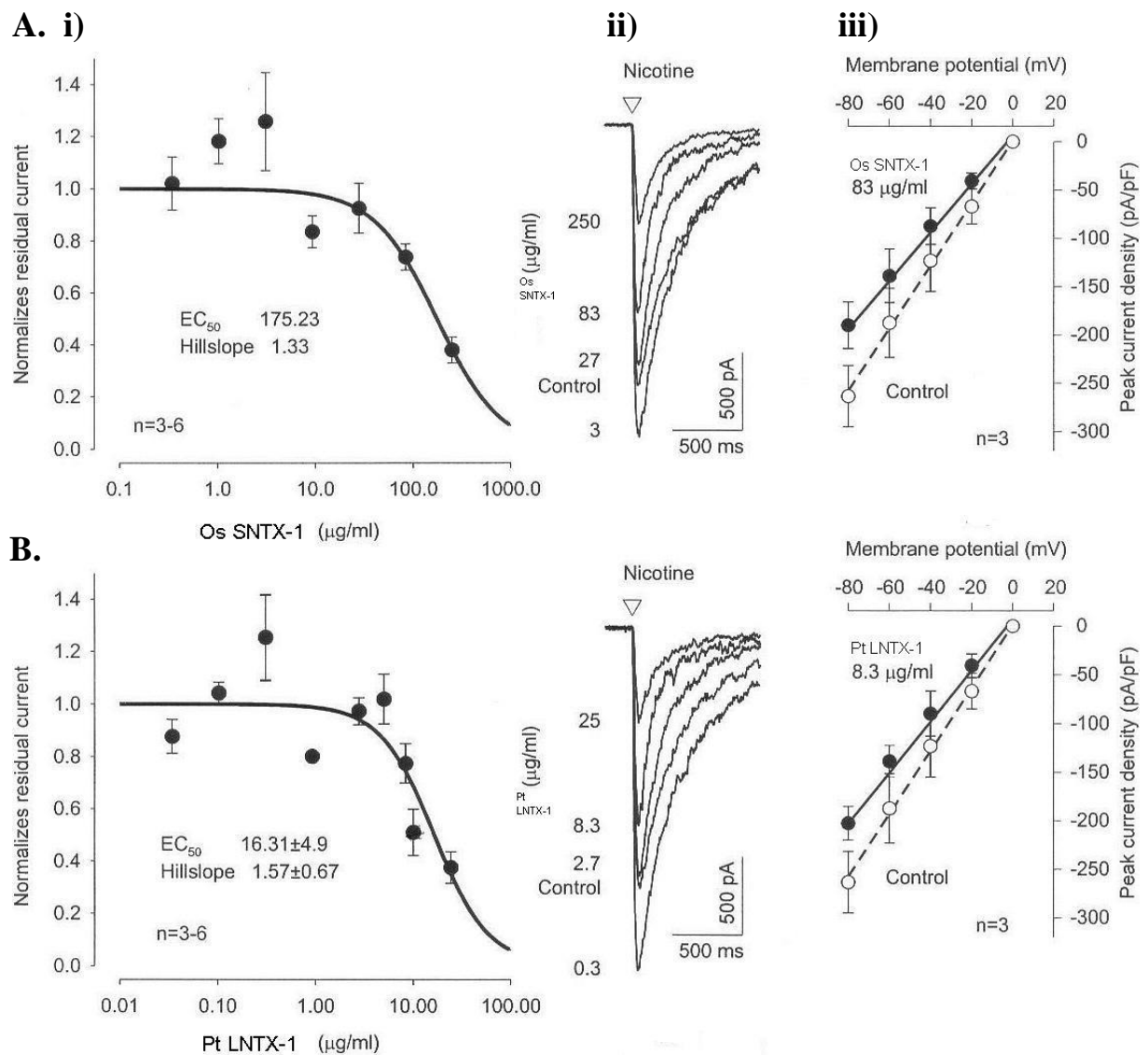
Figure 4.08. Concentration dependency of the recombinant neurotoxins in the electrophysiological assay.

The figure depicts results for **(A)** Os SNTX-1 and **(B)** Pt LNTX-1, and demonstrates **i)** concentration-response relationship for the inhibition of nicotine-evoked membrane currents from isolated bovine chromaffin cells by increasing the concentration of the recombinant neurotoxins (curves were applied on the basis of the equation appearing in methods and materials). **ii)** Superimposed nicotine (100μM)-evoked current traces recorded from isolated bovine chromaffin cells voltage clamped at -100mV in the absence and presence of different concentrations of neurotoxin (as stated). **iii)** Whole cell current-voltage relations obtained for peak current density (pA/pF) in the presence of neurotoxins (●) compared to controls (○).

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The functional activities of the coastal taipan SNTX-1 and common brown snake LNTX-1 recombinant proteins were further investigated via their effects on nicotine-evoked membrane currents in dissociated chromaffin cells. Bath application of increasing concentrations of both neurotoxins inhibited nicotine evoked depolarising membrane currents in cells voltage clamped at -100mV (Figure 4.08i). The initial stimulatory effect observed at low concentrations is a feature often observed for other toxins in this assay. High concentrations of toxin, however, almost totally blocked the activity of nicotinic acetylcholine receptors. This blockage was significantly greater for the Pt LNTX-1 recombinant toxin than Os SNTX-1, with a half maximal dose value of just 16μg/mL for Pt LNTX-1 compared to 175μg/mL for Os SNTX-1, confirming the results of the catecholamine release assay. The slope factor (Hill coefficient) of 1.33 and 1.57 for Os SNTX-1 and Pt LNTX-1 indicate that binding of both toxins to the nicotinic acetylcholine receptor occurs in approximately a 1:1 stoichiometry. Both recombinant neurotoxins depressed nicotine evoked current amplitude at all resting membrane potentials examined, although again, Pt LNTX-1 was more efficient at lower concentrations (Figure 4.08iii). This inhibition was voltage insensitive, with nicotine-evoked current amplitude reduced by ≥26% and ≥23% for all membrane potentials in the presence of 83μg/mL Os SNTX-1 or 8.3μg/mL Pt LNTX-1 respectively. This would indicate that both toxins are competitive antagonistic binders of the acetylcholine receptors, thereby inhibiting the binding of nicotine without activating the receptor, and not just blocking the channel and preventing intracellular cation exchange.

Discussion

Australian elapid snake envenomation results in a myriad of neurological effects within the bitten prey that are, in part, a result of the presence of postsynaptic neurotoxins that bind the nicotinic acetylcholine receptors of nerve and/or muscle fibres and inhibit their transmission. This study initially describes the identification of a short chain neurotoxin from the venom gland cDNA library of *O. scutellatus*, and its further characterisation by 5'-RACE and cloning. Sequencing identified this clone as a short chain neurotoxin which has previously been described at the mature protein level from the venom of this snake (Zamudio *et al.*, 1996). Subsequent PCR amplification identified a related family of toxins within the venom glands of six other Australian elapids, including the short neurotoxin Pa-a from *P. australis*

(figure 4.02, the first eight sequences) (Takasaki and Tamiya, 1985). Sharing 72.6% identity at the protein level, both Os SNTX-1 and Pa-a have previously been shown to be toxic to rats and mice with similar intravenous LD50 values of 0.063mg/kg and 0.076mg/kg respectively. Zamudio *et al*. reported an inability of native Os SNTX-1 to bind neuronal AChRs (typical of other short chain neurotoxins) and a reduced affinity for muscular AChRs, attributing this decrease in binding efficiency potentially to the substitution of either a phenylalanine or a histidine residue with a glycine residue at position 53 of the precursor molecule (figure 4.02). This substitution is also observed in the closely related *O. microlepidotus* molecule, Om SNTX-1, and would be predicted to have a similar effect. Interestingly, while the conserved histidine residue is observed in all other snake species for this particular family of neurotoxins, a second clone was also identified in the rough-scaled snake (*T. carinatus*) with a nonconservative amino acid substitution (H→N in Tc SNTX-1). A nonconservative substitution at this position may effect the efficiency of neurotoxin binding to the AChR as it occurs immediately adjacent to an arginine residue at position 54 of the precursor molecule that is conserved in all members of this family of proteins, including the sea snake and cobra sequences shown. This arginine residue has previously been shown to be crucial for optimal neurotoxin activity in earlier studies, and similarly site directed mutation of the amino acid immediately prior to this residue (that is at position 53 of the precursor molecule) of the short chain neurotoxin, erabutoxin a, resulted in a 7-fold reduction in the affinity for a muscular AChR (Pillet *et al.*, 1993). Phylogenetic analysis of these precursor protein sequences reveals that the Australian snakes demonstrate a distinct pattern of relatedness, with grouping similar to that of other venom gland specific genes described in this thesis (figure 4.03).

Interestingly, while PCR with primers designed from the original short chain neurotoxin identified from the *O. scutellatus* microarray chip identified a group of homologous proteins in related Australian elapids, there were two clones also amplified that showed little sequence relatedness. These included a second clone, Os SNTX-3, from *O. scutellatus* and the only short chain neurotoxin identified from *P. textilis*, Pt SNTX-8 (figure 4.02). A total of seven short chain neurotoxins have previously been cloned from the venom gland of *P. textilis*, and these highly related proteins represent a unique family of short chain neurotoxins (Gong *et al.*, 1999). Pt SNTX-8 is an eighth member of this family (most closely related to Pt SNTX-1) and overall there is a 74.7% degree of identity between the precursor protein sequences of these toxins. Functional studies revealed that these proteins exert the typical effects of short chain neurotoxins including high binding affinity with muscular AChRs resulting in muscle

paralysis, spasms and increased respiration, however their intravenous LD50 values in mice are much lower than other neurotoxins (Gong *et al.*, 1999). Variation between these sequences probably arises from point mutations, and the significant differences observed in primary structure (for example the loss of a proline from loop 1) between Pt SNTX-8 and the other neurotoxins may contribute to this decrease in lethality in a mammalian system. Mature Pt SNTX-8 has a theoretical molecular mass of 6345 Daltons, an exact match for the second of two native proteins purified by Gong *et al*. (1999) making it the closest match to one of these purified proteins.

Os SNTX-3 is a putative short chain neurotoxin that was identified by PCR along with Os SNTX-1 from *O. scutellatus* (figure 4.02). However Os SNTX-3 appears to be a completely novel sequence, not only within Australian elapids but also amongst any venomous animal. Zamudio *et al*. (1996) briefly alluded to the presence of a third neurotoxin within the venom of the coastal taipan with little sequence identity to toxins 1 and 2 which was completely ineffective in all nAChR assays performed. Hence this clone potentially warrants further characterisation in the future to determine its exact pharmacological role within the venom. The great variation observed in the primary sequence of neurotoxins within a single organism has been attributed to the variation, availability and susceptibility of prey, that is, a direct correlation between the deviation in venom composition with the target prey population and snake's diet (Daltry *et al.*, 1996). This is epitomised by the common brown snake, whose neurotoxic component pseudonajatoxin b is extremely lethal in a mammalian system, whilst the family of short chain neurotoxins have a reduced toxicity (Gong *et al.*, 2001; Gong *et al.*, 1999). However these proteins may have evolved specifically to target nAChR in other organisms, for example birds, frogs, lizards and other small reptiles, whose receptor binding sites may vary, providing the venom a selective arsenal of toxins to immobilise any prey of choice. This probably holds true of the variation observed in the neurotoxins (and indeed other proteins such as PLA_2s) observed within the coastal taipan and other related Australian elapids. The numerous low molecular bands observed in figure 4.05 further support the presence of multiple small toxic components within the venom of these snakes.

The secondary fold adopted by all postsynaptic neurotoxins is characterised by three adjacent loops rich in β-pleated sheets, held in a tight conformation by four disulfide bonds (refer to figure 1.03) (Kini, 2002; Walkinshaw *et al.*, 1980). Typically occurring as homodimers, single amino acid mutations within this framework are responsible for the variation in activity

and binding efficiency observed between toxins (Antil *et al.*, 1999). For example, the presence of a greater number of charged residues may affect the character of the molecular surface, or the presence of insertions or deletions change the size of the loops and hence interloop interactions (Tsetlin, 1999). However, a number of key conserved residues have been identified that are important for neurotoxin recognition and binding of the AChR. These include residues K27, W29, D31, R33 and L47 of the mature protein sequence of the postsynaptic short chain neurotoxin, erabutoxin, from the broad-banded blue sea krait, *Laticauda semifasciata* (Pillet *et al.*, 1993; Hori and Tamiya, 1976). The corresponding amino acids, K48, L50, D52, R54 and K68 of the precursor protein Os SNTX-1 are completely conserved, along with the 8 cysteine residues that form disulfide linkages (figure 4.02). Indeed these residues are almost completely conserved in all short chain neurotoxins identified in this study except for the two unusual isoforms Os SNTX-3 and Pt SNTX-8 observed in *O. scutellatus* and *P. textilis* respectively. This may indicate a decreased ability to bind nAChR or an alternate target site in these two proteins. The C termini of short chain neurotoxins always end in CNX, where X usually is N (Obara *et al.*, 1989; Tamiya *et al.*, 1985). This common sequence at the C terminus of the molecule, plus the high degree of conservation observed within the propeptide sequence, may explain why a single primer pair was able to detect multiple toxins between different snake species.

Along with the short chain neurotoxins, a number of unique long chain neurotoxin sequences were identified using a different primer pair (figure 4.04). All of these sequences represent their first description at the nucleotide level within an Australian elapid. Besides the increase in length, and often the presence of an additional disulfide bond located at the tip of loop 2, long chain neurotoxins are distinguished from short chain neurotoxins by their ability to bind and inhibit both muscular and neuronal AChRs (Servent *et al.*, 1998). The presence of a fifth disulfide bond has been implicated in this association with $\alpha 7$ homo-oligomeric neuronal AChRs (Servent *et al.*, 1998). However in mutagenic studies of α-cobratoxin from the venom of the monocled cobra, *Naja kaouthia*, both common and specific residues determined binding to the two receptor types. It was observed that the amino acids W25, D27, F29 R33, R36 and F65 in the mature protein were involved in binding to both neuronal and muscular AChRs (Antil *et al.*, 1999). However whilst A28, K35 and the C26-C30 disulfide bond were specifically involved in the association with $\alpha 7$ neuronal nAChRs, residues K232 and K49 solely bound to muscular nAChRs (Antil-Delbeke *et al.*, 2000). The corresponding residues

in the long chain neurotoxins identified in Australian elapids in this study are shown in figure 4.04.

Two of these sequences PA-ID from *P. australis* and Ns LNTX-1 from *N. scutatus* have previously been characterised at the protein level from the venom of these snakes (Takasaki, 1989; Halpert *et al.*, 1979). Whilst Ns LNTX-1 has been shown to be highly toxic in a mammalian system, PA-ID demonstrated an inability to bind nAChRs. The reason for this is evident when examining the key residues identified in α-cobratoxin that have been implicated in receptor binding. Almost all of these residues are conserved in Ns LNTX-1, whilst a number of substitutions are present in PA-ID (figure 4.04). This may indicate that PA-ID has evolved to target other specific receptor sites within different prey. The unique long chain neurotoxin Tc LNTX-1 cloned from *T. carinatus* contains the conserved fifth disulfide bond and many of the other residues implicated in AChR binding and therefore in theory should inhibit neural transmission in mammals. Interestingly, long chain neurotoxins were identified in the venom gland cDNA samples of *O. microlepidotus* and *O. scutellatus* which demonstrated significant homology to pseudonajatoxin b, a potently toxic (intravenous LD50 value of 0.15mg/kg) long chain neurotoxin from the venom of *P. textilis*. These sequences however lack the C-terminal extension present in pseudonajatoxin b. Some of the variable toxicity observed in other long chain neurotoxins arises from the diverse C-terminal extensions that distinguish them from short chain neurotoxins, particularly the presence of basic amino acid residues (Maeda and Tamiya, 1978). Also, notably, the clone Os LNTX-1 is missing the fifth disulfide bond, which is still present in Om LNTX-1 and -2. Again, this would indicate an inability to bind and inhibit α7 neuronal nAChRs. The presence of only four disulfide bonds in a long chain neurotoxin is unusual, but not unheard of, and does not necessarily result in loss of lethality (Kim and Tamiya, 1982).

Of all the long chain neurotoxins identified, Pt LNTX-1 from *P. textilis* was the most novel, sharing little identity with any other known venom neurotoxin (figure 4.04). Indeed, the closest match in databases searches is a neurotoxin from *L. colubrina* that shares only 51.4% identity at the mature protein level. While Pt LNTX-1 contains the conserved four disulfides common to the three finger toxins and highly similar propeptide sequence, it does contain a number of unique features. These include an alternative cleavage site for the signal peptide, the absence of the fifth disulfide bond as well as other deletions within the tip of loop 2 (amino acids 54 and 55 of the precursor) and other nonconservative substitutions within other

functionally important areas of the molecule. Hence, to determine the effects of these differences and the function of Pt LNTX-1 a recombinant protein was expressed in an *E. coli* expression system and analysed for activity. This included examining its ability to bind AChRs and inhibit catecholamine release, as well as a patch clamp study, which analyses the effects of a toxin on the flow of current across the cell membrane upon stimulation. For comparative analyses, a recombinant form of the short chain neurotoxin Os SNTX-1 from *O. scutellatus* was also produced and tested.

Investigation of both recombinant neurotoxins indicated their ability to inhibit the release of catecholamine from bovine adrenal chromaffin cells at high concentrations, although this inhibition was significantly greater for the Pt LNTX-1 toxin (figure 4.07). The reduced affinity Os SNTX-1 for nAChRs noted in this study is in keeping with the observations by Zamudio *et al*. (1999) for the native venom protein, which suggests that the pTWIN-1 expression system is an apt model for the production of a functionally active recombinant neurotoxin. Further investigation of both toxins suggests that they bind to the nAChR in a 1:1 stoichiometry (compared to the 2:1 stoichiometry observed in nicotine:receptor binding) (Figure 4.08). The patch clamp assay further identified both toxins as competitive agonistic binders of the nAChR, directly inhibiting the binding of nicotine without activating the receptor, and do not have a direct blockage effect of intracellular cation exchange. Interestingly, the novel long chain neurotoxin from the common brown snake had a significantly enhanced effect when compared to that of the short chain neurotoxin from the coastal taipan. This increased inhibitory effect probably arrises from differences in the primary structure between the two molecules as previously highlighted, as both contain only four inter-disulfide bonds, despite the toxin from *P. textilis* being a long chain neurotoxin. The decreased ability for Os SNTX-1 to bind a mammalian receptor may again be indicative of the snake's requirement for multiple toxin isoforms to accommodate for varying receptor types in different prey species. Hence this study has described and partially characterised a novel long chain neurotoxin from the common brown snake, which given its unique sequence at the primary amino acid level and activity within a mammalian system, warrants further investigation in the future.

A 21 amino acid signal peptide was identified for all clones, often representing the first description of this sequence for not only novel neurotoxins, but also neurotoxins previously described at the mature protein level. This sequence was observed to be highly conserved not

only within the short chain and long chain neurotoxins, but also between long and short chain neurotoxins, indicating a common pathway for the folding and secretion of a functional protein. This high degree of conservation within the propeptide sequence is a feature observed in all other secreted toxins characterised in this thesis. This leader sequence contains a high proportion (approximately 60%) of hydrophobic residues, consistent with its role as a secretion signal. Previous studies have indicated that the presence of a cysteine residue at position 15 of the precursor neurotoxin is known to influence the rate of folding of the mature protein (Bouet *et al.*, 1982; Menez *et al.*, 1980). This cysteine residue is completely conserved in all long and short chain neurotoxin sequences reported in this study with the exception of the clone Ns SNTX-1 from *N. scutatus*, which has possible implications for the correct folding and processing of this molecule.

It is evident from the results of this and previous studies that snake venoms are a source of multiple isoforms of neurotoxins and other proteins. It is postulated that different neurotoxins from a single venom source may exert different physiological effects depending on the target prey (Phui Yee *et al.*, 2004). By maintaining the robust three-finger protein mould, it has been possible for the snake to recruit an arsenal of toxins with a wide variation of function and activity by just a few subtle changes to residues within functional sites (Kini, 2002). It has also been suggested that the postsynaptic neurotoxins and other venom proteins have undergone a process of accelerated evolution via directional mutation to allow for this adaptation (Afifiyan *et al.*, 1999; Ogawa *et al.*, 1992). Hence, selection pressure may have favoured the multiplicity of isoforms observed in short and long chain neurotoxins. Some animals, for example the mongoose, contain mutated AChRs that are resistant to the binding of α-neurotoxins, and hence it is the heterogeneity of these target receptors that influence toxin evolution (Barchan *et al.*, 1995; Barchan *et al.*, 1992). Alternatively, recent evidence suggests that snakes that no longer require venom for the capture of prey due to a diet exclusively consisting of fish eggs contain a truncating mutation within their neurotoxin, which results in the loss of function of that protein (Li *et al.*, 2005). Hence, variations observed in this study in the isoforms of short and long chain neurotoxins probably reflect variable physiological effects of these proteins as dictated by the snakes diet.

In conclusion, this study describes the cloning and partial characterisation of a number of novel long and short chain neurotoxins from the venom glands of eight venomous Australian snakes. It represents the most detailed study of the postsynaptic neurotoxic components from

the venom of these elapids at the molecular level to date. It is hoped that results of the study will firstly provide information on the mechanisms of action of these venom components (and hence aid in the treatment and detection of snake bite), secondly, expand our understanding of the evolutionary relationships of Australian elapids, and finally provide research tools for the future development of molecules that may be applied in either diagnostic or therapeutic environment.

CHAPTER 5

Identification and Characterisation of Venom Natriuretic Peptides from Australian Elapids

Snake venoms are a unique source of toxins that exert specific and targeted effects within mammalian systems. Amongst these diverse proteins are the venom natriuretic peptide family, which include small molecules (approximately 40 amino acids in length) identified from the venoms of a number snakes including elapids and vipers (Fry *et al.*, 2005; Amininasab *et al.*, 2004). Although studies have indicated that native venom natriuretic peptides are potent simulators of natriuresis, diuresis and vasorelaxation, their exact role within the venom has yet to be fully elucidated (Best *et al.*, 2002; Lisy *et al.*, 1999). Members of the venom natriuretic peptide family are grouped on the basis of their structural and functional similarity to mammalian natriuretic peptide counterparts. These proteins are characterised by the presence of a 17 amino acid disulfide bound ring and have a significant role in a number of physiological processes including cardiovascular, renal and endocrine homeostasis (Chen and Burnett, 2000).

The mammalian natriuretic peptide family contains a number of proteins that differ not only in their primary structure, but also their tissue of origin and receptor targets (Misono, 2002). Both atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are produced in myocardial cells (although BNP was originally isolated from porcine brain, hence the nomenclature), and both bind the natriuretic peptide-A receptor (NPR-A), thereby stimulating the second messenger cyclic GMP system for flow on effects (Sudoh *et al.*, 1988). A third member of the family, C-type natriuretic peptide (CNP), lacks the C-terminal extension present in the other proteins and is expressed predominantly within the central nervous system and vascular endothelial cells. Binding to natriuretic peptide-B receptors (NPR-B), its effects are strongly vasoactive, with little effect on natriuresis (Koller *et al.*, 1991). A third type of receptor (NPR-C) has also been identified and shown to bind all three natriuretic peptide subtypes, and has been implicated in the control of the local concentration of natriuretic peptides available to the other receptors (Maack, 1992).

The most widely studied of the venom natriuretic peptides is Dendroaspis natriuretic peptide (DNP), isolated from the green mamba, *Dendroaspis angusticeps* (Schweitz *et al.*, 1992). DNP is a 38 amino acid protein that demonstrates structural features, receptor binding

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characteristics, second-messenger activity and some vasoactive effects similar to that of ANP and BNP (Richards *et al.*, 2002). The gene encoding for this peptide has yet to be determined. More recently, a group of natriuretic peptides have been isolated from the venom of the Australian elapid genus, *Oxyuranus* (Fry *et al.*, 2005). Three peptides, TNP-a, TNP-b and TNP-c, have been isolated from the venom of the inland taipan, *O. microlepidotus*. All three of these proteins have also been identified in the Papuan taipan, *Oxyuranus scutellatus canni*, whilst the first two peptides were isolated from the coastal taipan *O. scutellatus*. These peptides were 35-39 amino acids in length (3.5kDa to 4kDa) and contained conserved cysteine residues typical of the 17 amino acid ring structure observed in natriuretic peptides, with a novel C-terminal tail. Upon pharmacological analysis, the three peptides demonstrated variable vascular and natriuretic activities, resulting from minor variations in primary structure.

Multiple properties of the natriuretic peptides, including their effects on diuresis, natriuresis, vasorelaxation and antimitogenesis, make them ideal candidates for pharmacological therapy of cardiovascular disease (Pan *et al.*, 2004; Colucci *et al.*, 2000). Target disorders that have so far been identified as potentially benefiting from intervention with a synthetic natriuretic peptide derivative include congestive heart failure, hypertension and ischemic heart disease (Han and Hasin, 2003). The aim of this study was to further characterise a venom natriuretic peptide transcript identified from screens of the *O. scutellatus* microarray via cloning and recombinant protein expression, as well as perform a comparative analysis with homologous transcripts from related Australian elapids. It is hoped that comparative analysis and characterisation of this family of proteins will provide significant information on their role within the venom as well as contribute to the development of natriuretic peptides as a new class of therapeutics.

Methods and Materials

RNA Isolation and cDNA Synthesis

RNA was isolated and cDNA synthesised from Australian elapid venom glands as previously described. Again, the snakes involved in this study included the coastal taipan (*Oxyuranus scutellatus*), inland taipan (*Oxyuranus microlepidotus*), common brown snake (*Pseudonaja*

textilis), red-bellied black snake (*Pseudechis porphyriacus*), mulga (*Pseudechis australis*), mainland tiger snake (*Notechis scutatus*), rough-scaled snake (*Tropidechis carinatus*) and Stephen's banded snake (*Hoplocephalus stephensii*).

Identification of Venom Natriuretic Peptides

An mRNA transcript identified from screens of the microarray included a clone coding for the mature sequence of a venom natriuretic peptide. This sequence was further identified in *O. scutellatus* by 5'-RACE and subsequently PCR amplified and cloned from the other related snake species. 5'-RACE was performed as previously described with a gene specific primer (5'-GGA CGA GTC TGT TGC AGC CCA GGC CGC T-3'), and the resulting 200bp product cloned and sequenced.

The coding sequence of the mature peptide was then amplified by PCR from venom gland cDNA from all Australian elapids using primers designed from the original library clone and the 5'-RACE product. The 25μL reaction mixture contained approximately 200ng of venom gland cDNA template, 1 unit of AmpliTaq gold buffered in 10mM Tris-HCl pH 8.3, 50mM KCl, 1.75mM $MgCl_2$ and 200μM dNTPs with 25ρmol of each of the forward (5'-CCG CGG CTG GGC TCA G-3') and reverse (5'-CCA TTT TCA GCT TAC CCC C-3') primers. The reaction was thermocycled at 95°C for 10min, followed by 27 cycles of 95°C for 20sec, 55°C for 20sec and 72°C for 90sec, with a final extension of 72°C for 3min. The PCR products were run on a 1% TAE agarose gel, a single band excised corresponding to each snake and multiple clones sequenced as previously described. Alignments were then performed of the deduced amino acid sequence for each unique clone using BioEdit software.

Subcloning and Expression of Recombinant Venom Natriuretic Peptides

Two venom natriuretic peptides were selected for further characterisation via recombinant expression and functional analysis. These included the unique clone from *P. textilis*, PtNP-a, and the most distantly related clone from *P. australis*, PaNP-c. Hence, full-length cDNA clones corresponding to both of these proteins were subcloned into the pTWIN1 vector for transfection and recombinant expression as previously described (New England Biolabs,

Beverly, Massachusetts). Both constructs were prepared in their native form without the addition of any extra amino acid residues.

A 50μL reaction was prepared with approximately 50ng of natriuretic peptide in pGEM-T vector as template for both constructs, 2 units of AmpliTaq gold buffered in 10mM Tris-HCl pH 8.3, 50mM KCl and 1.5mM $MgCl_2$ with 200μM dNTPs and 50ρmol of each of the forward and reverse primer. PtNP-a was amplified with the forward primer (5'-GGT GGT TGC TCT TCC AAC AGC GGC TCC AAG ATA AGG GA-3') whilst PaNP-c was amplified with the forward primer (5'-GGT GGT TGC TCT TCC AAC AGC GGC TCC AAG ACG GCC-3'). Both PCR reactions used a common reverse primer with Sap I restriction sites included (5'-GGT GGT TGC TCT TCC GCA TTA GGA TCC GCC AGG TCT-3'). The reactions were thermocycled initially at 95°C for 8min, then 30 cycles of 95°C for 20sec, 58°C for 20sec and 72°C for 30sec with a final extension of 72°C for 5min. The PCR product was then precipitated, quantitated, digested with Sap I, ligated and cloned into pTWIN1 all as previously described in chapter 4. Clones were sequenced to confirm the presence of insert and transformed into electrocompetent BL21 *E. coli*.

Recombinant PtNP-a (rPtNP-a) and rPaNP-c constructs in BL21 were grown to an O.D. of 0.5 from an overnight starter culture in the presence of 100μg/mL of ampicillin at 37°C. Protein expression was induced with 0.5mM IPTG, shaking at 15°C overnight and cells harvested by centrifugation. Recombinant protein was affinity purified on chitin beads as previously described and target protein cleaved via a decrease in pH and an increase in temperature. Purified recombinant protein products, as well as samples of induced and uninduced cell lysate, washes and the chitin beads, were then analysed on a 16.5% SDS-PAGE stained with coomassie as previously described. All eluted fractions were then pooled for both venom natriuretic peptides and loaded onto a prewashed, 10kDa molecular weight cut-off protein column. Purified recombinant protein was collected in the flow through, with any residual contaminating proteins and chitin tag removed via exclusion on the basis of size. Samples of the flow through and the residual volume retained by the column were then examined on a 16.5% SDS-PAGE, and the final concentration of both proteins quantitated via a Lowry assay as previously described

Angiotensin-Converting Enzyme Inhibition Assay

The rPtNP-a and rPaNP-c constructs were then sent to the Faculty of Health Sciences department of the University of Queensland at the Princess Alexandra Hospital for functional analysis by Dr Simone Flight. Both proteins were concentrated by centrifugation under vacuum and their molecular masses determined by matrix assisted laser desorption/ionisation - time of flight (MALDI-TOF) mass spectrometry analysis. The ability of the recombinant peptides to inhibit the activity of angiotensin-converting enzyme (ACE) was then examined using a continuous spectrophotometric assay first described by Holmquist *et al.* (1979) and later modified by Buttery and Stuart (1993). Briefly, the activity of ACE was determined by the conversion of furylacrylolphenylalanylglycylglycine (FAPGG) to FAP and glyclglycine. The concentration of FAPGG can be determined by spectrophotometric absorbance at 345nm, decreasing upon cleavage with ACE. ACE activity was determined with 0.0075U of enzyme in a 1mL reaction volume with 1mM FAPGG in borate buffer pH 8.2, after a pre-incubation for 5min. Recombinant natriuretic peptide constructs were then added at varying concentrations and the rate of ACE activity again determined spectrophotometrically. The percentage inhibition of ACE could then be calculated by comparison to normal rate of ACE activity. All results were imported into Microsoft Excel and graphed accordingly.

Results

A single 497bp clone was isolated from the *O. scutellatus* venom gland cDNA array whose predicted protein sequence at the 5' end was identified as a natriuretic peptide by database homology search. This transcript only extended two nucleotides beyond the first codon of the mature protein, hence an internal primer was designed within this sequence for 5'-RACE. It was only possible to obtain a 200bp product by 5'-RACE, which was cloned and sequenced (figure 5.01A). As the mature protein contains a serine residue at its first position, presumably the natriuretic peptide contains a propeptide like every other venom protein. The cleavage site between this propeptide and mature protein was found to be PAAGL↓SDP by 5'RACE in the coastal taipan. It was not possible to obtain a longer 5'RACE product, even though a methionine residue was not reached. Using primer sequences designed within this small leader sequence and within the 3'UTR, a PCR product corresponding to the mature natriuretic peptide was amplified in all other Australian elapids (figure 5.01B).
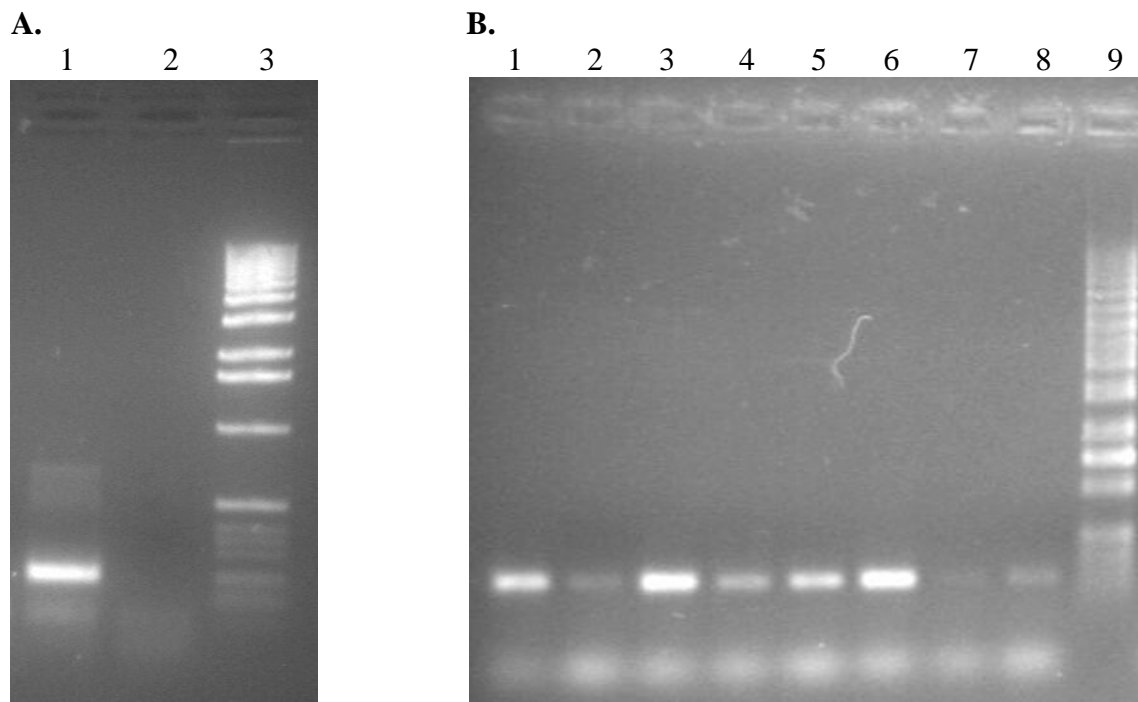
Figure 5.01. Identification of natriuretic peptides.

(A) A single 5'-RACE product from the venom gland cDNA of *O. scutellatus* corresponding to a natriuretic peptide (lane 1) compared to a negative control (lane 2) and molecular weight marker. **(B)** PCR amplification of a transcript coding for the mature protein sequence of venom natriuretic peptides from 1) *O. scutellatus*, 2) *P. textilis*, 3) *O. microlepidotus*, 4) *P. porphyriacus*, 5) *P. australis*, 6) *T. carinatus*, 7) *N. scutatus*, 8) *H. stephensii* and 9) molecular weight marker.

All PCR products were cloned and sequenced. An alignment of the deduced mature protein sequence of all unique clones is shown in figure 5.02. In many instances, multiple isoforms of venom natriuretic peptides were identified from the one snake, particularly within *P. australis* in which four different transcripts were identified. A single transcript was identified from *O. scutellatus* (OsNP-d) that, although demonstrating significant identity to the three proteins isolated by Fry *et al*. (2005), was still a different protein (figure 5.02). Interestingly, identical proteins were also cloned from the venom gland cDNA of the closely related species *O. microlepidotus* (OmNP-d) and *P. australis* (PaNP-a). These proteins were also longer than those previously identified in the taipans. The cysteine residues involved in the formation of the 17 amino acid disulfide bound ring were completely conserved between all species, and the area of greatest similarity lies between these two residues. Note that all sequences share little identity with DNP from the green mamba, *Dendroaspis angusticeps*, outside of the

primary ring structure (figure 5.02). It is also of interest that the *H. stephensii*, *N. scutatus*, *T. carinatus*, *P. porphyriacus* and half of the *P. australis* sequences contain a 3 amino acid insertion (K/E, T/V, A) at the N-terminus and a 3 to 4 residue deletion within the C-terminal region of their venom natriuretic peptides. Multiple proline residues are also evident in the C-terminal tail of all proteins. Residues that have previously been shown to play a key role in the binding of mammalian atrial natriuretic peptides to their receptors also demonstrate a reasonably high degree of conservation with the snake proteins (Oikawa *et al.*, 1984).

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                                 10        20        30        40
                            ....|....|....|....|....|....|....|....|...
TNP-a                     1 SDS---KIGDGCFGLPLDHIGSVSGLGCNRPVQNRPKK----- 35
TNP-b                     1 SDP---KIGDGCFGLPLDHIGSVSGLGCNRPVQNRPKK----- 35
TNP-c                     1 SDS---KIGNGCFGFPLDRIGSVSGLGCNRIMQNPPKKFSGE- 39
O. scutellatus OsNP-d     1 SDP---KIGNGCFGFPIDRIGSVSGLGCNRLVQNPPKPISGES 40
O. microlepidotus OmNP-d  1 SDP---KIGNGCFGFPIDRIGSVSGLGCNRLVQNPPKPISGES 40
O. microlepidotus OmNP-e  1 SDS---KIGDGCFGLPLDHIGSVSGLGCNRPVQNRPKQIPGGS 40
P. textilis PtNP-a        1 SGS---KIGNGCFGLPLDRISNTSGMGCRNPIQNRPKSTPGGS 40
P. australis PaNP-a       1 SDP---KIGNGCFGFPIDRIGSVSGLGCNRLVQNPPKPISGES 40
P. australis PaNP-b       1 SDS---KIGDGCFGLPLDHIGSVSGLGCNRPVQNRPKQIPGGS 40
P. australis PaNP-c       1 SGSKTAEIGDGCFGVPIDHIGSTSGMGCGRP---RPKPTPGGS 40
P. australis PaNP-d       1 SGSKTAEIDDGCFGLPLDPIGSTSGMGCRS----VPKPIPGGS 39
P. porphyriacus PpNP-a    1 SGSKTANIGDGCFGVPIDHIGSTSGMGCGSP---RPKPTPGGS 40
P. porphyriacus PpNP-b    1 SGSKTANIGDGCFGVRLDHIGTTSGMGCGRP---RPKPTPGGS 40
T. carinatus TcNP-a       1 SGSETAKIGDGCFGLPIDRIGSASGMGCGS----VPKPTPGGS 39
N. scutatus NsNP-a        1 SGSEVAKIGDGCFGLPLDRIGSASGMGCRS----VPKPTPGGS 39
N. scutatus NsNP-b        1 SGSKTAKIGDGCFGLPLDRIGSTSGMGCGS----VPKPTPGGS 39
H. stephensii HsNP-a      1 SGSKTAKIGDGCFGVRLDRIGSTSGMGCGG----VPKPTPGGS 39
H. stephensii HsNP-b      1 SGSKTAKIGDGCFGVPIDHIGSTTDLGCGRP---RPKPTPRGS 40
DNP                       1 -----EVKYDPCFGHKIDRINHVSNLGCPSLRDPRPNAPSTSA 38
ANP                                       FG   M RI      L
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Figure 5.02. Protein alignment of venom natriuretic peptides

Sequences identified from the venom gland of 8 Australian elapid snakes (DQ084065; DQ116722 – DQ116735) are compared to those previously isolated from the *Oxyuranus* species TNP-a (P83224), TNP-b (P83227) and TNP-c (P83230), as well as DNP (AAB22476) from *Dendroaspis angusticeps*. Dotted arrows denote the 17 amino acids that form the disulfide-bound peptide ring, including conserved cysteine residues. Residues previously shown to be important in receptor binding by *H. sapiens* ANP (P01160) are also shown.

To further investigate the role of natriuretic peptides in the venom of Australian elapids, two of the above proteins were selected for recombinant expression and functional analysis. Although both contained conserved residues implicated in the formation of the 17 amino acid ring structure common to natriuretic peptides, these two constructs only shared 65.1% identity

at the mature protein level. The pTWIN1 *E. coli* expression system was employed to produce a recombinant form of each protein without the addition of any tags or residual amino acids. Figure 5.03A demonstrates the expression and purification of one of these constructs, recombinant PtNP-a (rPtNP-a). Induction of protein expression and efficient cleavage from the chitin/intein tag upon a pH and temperature shift was observed for both proteins. The four elution volumes were then pooled and run through a 10kDa molecular weight cut-off column to remove any residual tag or contaminating proteins. The highly pure recombinant protein is evident in figure 5.03B (lanes 2 and 5), with contaminating tag and proteins retained by the column (and effectively concentrated, hence the presence of additional bands in lanes 3 and 6 which weren't originally evident in the first elution, lanes 4 and 7). The final concentration of each protein was calculated to be 269µg/mL for rPtNP-a and 257µg/mL for rPaNP-c. Hence it was possible to obtain approximately 1.5mg of purified protein per litre of original bacterial culture without the necessity of multiple purification steps.

Both recombinant protein constructs were then concentrated and their masses independently determined by MALDI-TOF mass spectrometry. The calculated molecular weights of 4061 Daltons for rPtNP-a and 3872 Daltons for rPaNP-c correlate well with the theoretical molecular masses for both proteins, which were determined to be 4062 Daltons and 3872 Daltons respectively. Angiotensin-converting enzyme (ACE) catalyses the conversion of angiotensin to angiotensin II, a vasoconstrictive peptide. ACE plays an integral role in the renin-angiotensin system (Lumbers, 1999). Natriuretic peptides previously identified in mammals have been shown to inhibit the renin-angiotensin system, resulting in vasorelaxation and hence pose a target as potential therapeutics (Cho *et al.*, 1999). To examine the effects of the venom natriuretic peptides identified and expressed in this study on ACE, their activities were analysed by a standard continuous spectrophotometric assay, which measures the cleavage of the substrate FAPGG by ACE. Both the *P. textilis* PtNP-a and *P. australis* PaNP-c recombinant proteins resulted in a dose dependent inhibition of ACE activity (figure 5.04). Approximately 50% of ACE inhibition was observed at 1µM concentrations for both peptides. This inhibitory effect on ACE would be predicted to result in vasorelaxation in an *in vivo* setting.
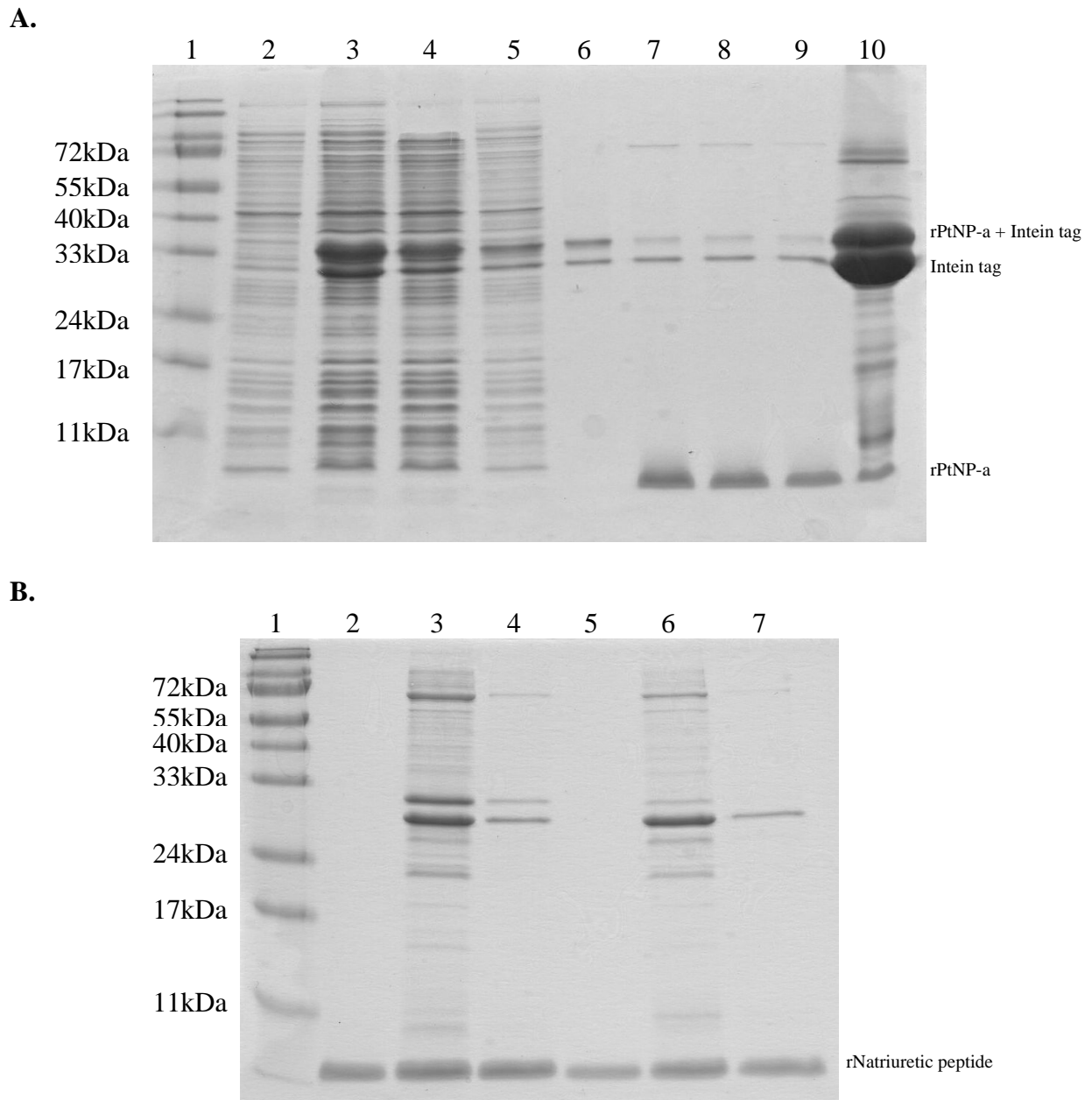
Figure 5.03. Recombinant natriuretic peptide purification.

(A) Purification of recombinant *P. textilis* PtNP-a loaded on a 16.5% SDS-PAGE stained with coomassie. Lanes were loaded as follow: 1) Molecular weight marker, 2) Uninduced cell lysate, 3) Induced cell lysate, 4) Unbound cell lysate, 5) Column wash 1, 6) Column wash 4, 7) Elution 1, 8) Elution 2, 9) Elution 3 and 10) Bound chitin beads. Note the presence of the cleaved recombinant protein in the elutions, along with residual chitin/intein tag. **(B)** Purification of pooled recombinant natriuretic peptide elutions by size exclusion on a 10kDa molecular weight cut-off column. Lanes were loaded as follows: 1) Molecular weight marker, 2) Purified rPtNP-a, 3) Residual rPtNP-a, 4) rPtNP-a elution 1, 5) Purified rPaNP-c, 6) Residual rPaNP-c and 7) rPaNP-c elution 1.

173
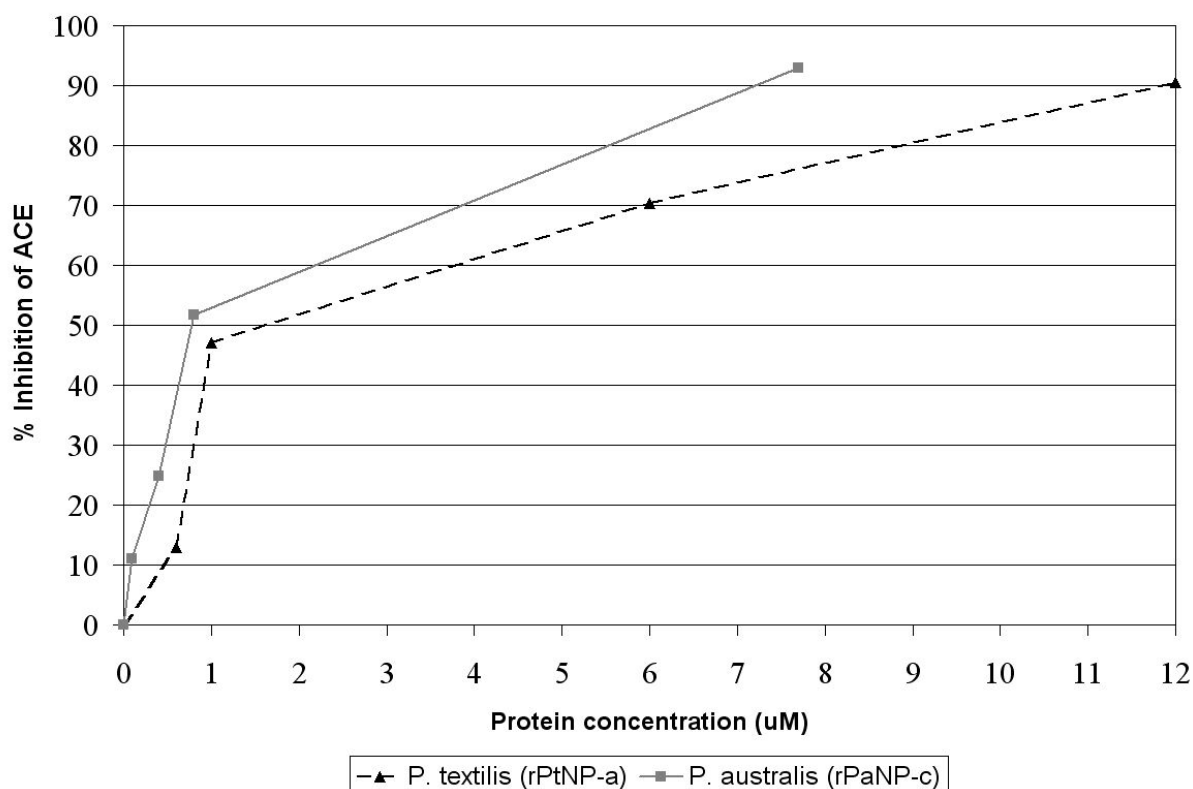
Figure 5.04. Angiotensin-converting enzyme inhibition assay results.

Graph depicting the dose dependent inhibition of ACE by the recombinant natriuretic peptides PtNP-a and PaNP-c from *P. textilis* and *P. australis* respectively.

Discussion

Natriuretic peptides are a distinct family of proteins classified on the basis of their primary structure, which includes a 17 amino acid disulfide enclosed ring. They have been isolated from a number of tissue sources including mammalian myocardial and endothelial cells and have been shown to mediate a number of physiological processes including natriuresis, vasodilation, renin-angiotensin system inhibition and antimitogenesis (Chen and Burnett, 1999). Over the past decade the presence of natriuretic peptides has been detected in the venom of a number of snakes, including its first description, DNP, in the green mamba, *Dendroaspis angusticeps* (Schweitz *et al.*, 1992). Recently, Fry *et al.* (2005) reported the presence of three novel natriuretic peptide amino acid sequences from the venom of the Australian snake genus *Oxyuranus*, and the activity of one of these peptides (TNP-c) in assays measuring relaxation of rat aortic rings and elevation of cyclic GMP. The study described

here details for the first time cDNA sequences of natriuretic peptides from the venom gland of an Australian elapid, which were found to occur as multiple isoforms within many of these snakes.

Although a single natriuretic peptide clone identified in this study was common to both the inland and coastal taipan (OmNP-a and OsNP-a respectively), this protein differed from all three sequences described by Fry *et al*. (2005) (figure 5.02). Of these three peptides, only TNP-c was shown to be equipotent to atrial natriuretic peptide (ANP) and DNP in producing a near total relaxation of pre-contracted rat aortae, despite significant identity at the primary amino acid level. However a number of key differences were observed between TNP-c and TNP-a and -b, which may contribute to these observed functional differences. Most notably is the substitution within the loop of an invariant arginine residue at amino acid position 16 with a histidine residue that may affect interactions with the mammalian natriuretic peptide receptor (He *et al.*, 2001). Interestingly, this alteration was observed in a number of the Australian elapid sequences identified in this study, along with a unique substitution of the arginine with a proline residue in PaNP-d from *P. australis*. This variation in the isoforms of natriuretic peptides within and between snakes reported in this study may reflect the variation, availability and susceptibility of prey, that is, a direct correlation between the deviation in venom composition with the target prey population and snake's diet (Daltry *et al.*, 1996). It has previously been postulated that the numerous neurotoxin and phospholipase A_2 isoforms observed in snake venoms have evolved so as to effect the many different targets presented by different prey organisms (which may include reptiles, mammals, amphibians and birds), and this may also be true of the venom natriuretic peptide family (Kordis and Gubensek, 2000). Another major difference observed between the TNP-c and TNP-a and -b was the size of the C-terminal tail, a region of the protein which has previously been shown to have important implications for the function of ANP (Watanabe *et al.*, 1988). The C-terminal tails of the sequences described from Australian snakes in this study were longer then those of ANP and the TNPs, and contained a high proportion (either 3 or 4 residues) of prolines. The location and number of these proline residues may also alter the activity of individual molecules.

Beside the residues described above, a number of other conserved amino acids have been identified from mammalian natriuretic peptides that are important in the binding and stimulation of the natriuretic peptide receptor. These include phenylalanine, glycine, methionine, isoleucine and leucine residues within the 17 amino acid ring of ANP at the

positions depicted in figure 5.02 (Thibault *et al.*, 1995; Watanabe *et al.*, 1988; Wakitani *et al.*, 1985). It is evident that the F10, G11 and I17 of OsNP-d from *O. scutellatus* are completely conserved between all Australian elapid snake sequences, DNP from the green mamba as well as human and rat ANP and BNP, confirming the importance of these residues in the functional activity of natriuretic peptides. Similarly, the methionine and isoleucine residues of ANP, corresponding in position to I14 and L23 of OsNP-d respectively, are replaced by other conserved hydrophobic residues (methionine, leucine and isoleucine) and would not be expected to alter the function of the molecule. Hence, given the conservation of these residues, together with the cysteines that form the 17 amino acid ring structure, the natriuretic peptides from Australian snakes would also be predicted to have a vasoactive effect within a mammalian system, and the variation observed in other areas of the molecule have probably arisen to account for the different receptor subtypes encountered by the snake in different prey.

Although a single clone was identified from the screens of the microarray corresponding to the mature protein sequence of a venom natriuretic peptide, 5'-RACE analysis failed to identify the entire propeptide for this molecule, just the final six amino acids prior to the cleavage site. Human ANP is synthesised in the atrial myocyte as a 151 amino acid prohormone with a 25 amino acid signal peptide that is cleaved from the N-terminus within the endoplasmic reticulum (Stoupakis and Klapholz, 2003). Given the absence of a methionine residue at the N-terminus of the *O. scutellatus* protein, the venom natriuretic peptides from Australian elapid snakes probably also posses a signal peptide, as do all other toxins characterised in this thesis, which may be the subject of further study in the future.

Two of the most distinct natriuretic peptides identified in this study were then selected for recombinant expression and preliminary functional analysis. Constructs containing PtNP-a from *P. textilis* and PaNP-c from *P. australis* were produced in an *E. coli* expression system that had the duel advantage of being able to rapidly produce high quantities of protein absent of any tag or additional amino acids with a purification system that does not contain any reducing agents, which means that the 17 amino acid ring, if formed, should remain intact. Both recombinant constructs were observed to exhibit a direct dose-dependent inhibition of the activity of angiotensin-converting enzyme (ACE) (figure 5.04). Rates of inhibition were comparable (slightly greater in rPaNP-c) despite these proteins only sharing approximately 65% identity. ACE is a carboxypeptidase that plays an integral role in the renin-angiotensin

system, responsible for the conversion of angiotensin to angiotensin II whose flow on effects include vasoconstriction via the stimulation of the sympathoadrenal system, natriuretic effects and direct regulation of aldosterone secretion (Lumbers, 1999). ACE inhibitors are amongst the most potent antihypertensive drugs currently available (Toto *et al.*, 2004). Hence, the recombinant natriuretic peptides described here provide a useful platform for further investigation of venom proteins as antihypertensive/congestive heart failure agents. Similarly, by subjecting recombinant proteins to an array of functional tests an as yet unknown function may be assigned to these molecules that can then be exploited in a therapeutic or diagnostic setting.

The exact role of natriuretic peptides within the venom of snakes still remains unclear. Their effects may be targeted towards increasing vascular permeability so as to aid the systemic delivery of other toxic components within the venom. Similarly, ACE inhibition may also contribute to the post-envenomation hypotensive effect thereby aiding immobilisation of prey, however there may be some other as yet unelucidated activities of these proteins that play an important functional role within the venom. In summary, this study has described for the first time, the cloning, expression and characterisation of members of the natriuretic peptide family from the venom of a number of Australian elapid snakes.

CHAPTER 6
General Discussion

Snake venoms are a complex mixture of polypeptide and other molecules that specifically target a variety of physiological systems within their prey in an adverse manner. The venom of Australian snakes, which belong almost exclusively to the elapid family, are amongst the most toxic in the world in intravenous LD50 studies (Broad *et al.*, 1979). Their effects explicitly target the neuronal, neuromuscular and cardiovascular systems of envenomated prey in a highly specific manner (Fry, 1999). These venoms may contain upwards of a hundred proteins that, although demonstrating significant structural homology, display a wide variety of functions. Given the relatively high degree of toxicity of Australian elapid venoms, there has been a significant amount of research into the biochemical and physiological properties of their constituents and the venom as a whole, but comparatively few studies into these toxins at the molecular level (Tan and Ponnudurai, 1990). Research has, until recently, focussed on three primary classes of molecules from elapid venoms: the prothrombin activating enzymes, the neurotoxins and the phospholipase A_2 enzymes. Evidence suggests that Australian snake venoms contain numerous other molecules, including natriuretic peptides, ion channel blockers, L-amino acid oxidases and plasmin inhibitors and may still as yet be the source of other unique, unidentified proteins (Fry *et al.*, 2005; Masci *et al.*, 2000; Brown *et al.*, 1999; Stiles *et al.*, 1991).

Given the apparent diversity of toxins of varying structure and function within the venom of snakes, this study was performed for the systematic comparative analysis of toxin sequences at the level of cDNA in a cohort of eight Australian elapids. It represents the most comprehensive study of the constituents of Australian snake venoms at the molecular level to date. A detailed molecular analysis is the first step in determining the structural-functional relationship of venom components, which may then be applicable to a number of pharmacological settings, including their use as potential diagnostic or therapeutic agents, or in the detection and treatment of snake bite. To this end, the primary aims of this study were two-fold. Firstly, to further characterise a prothrombin activating protein already targeted as a potential therapeutic agent, including cDNA cloning, phylogenetic analysis, detection in venom and characterisation of the recombinant protein. The second aim of this project was the identification and comparative analysis of novel components from the venom gland of the coastal taipan and related Australian elapids, via the production and screening of a cDNA

microarray. Performing a molecular/cDNA analysis of venom components has a number of distinct advantages. It is a much more rapid and accurate way to identify all isoforms of a family of toxins than the purification and sequencing of native protein from the venom. DNA sequences also provide information on not only the phylogenetic association between related snakes, but also on the evolution of the toxin itself (Mebs, 2001). Finally, a number of methodologies exist for the recombinant expression of proteins, or parts thereof, allowing for the determination of the function of a specific toxin or domain within that toxin.

Haemostatic Properties of Australian Snake Venoms

One of the distinguishing features of Australian elapids compared to all other venomous species of snake is that the procoagulant effects of their venom are limited to prothrombin activation alone: they contain no thrombin-like enzymes (Chester and Crawford, 1982). This is due to the presence of prothrombin activators within the venom that are structurally and functionally similar to mammalian blood coagulation factor Xa (Markland, 1998). One such factor Xa-like prothrombin activator from the venom gland of the common brown snake (*P. textilis*) has been targeted as a potential surgical tissue adhesive to promote haemostasis and tissue sealing during surgery (Jackson, 2001; Masci *et al.*, 1988). This study has identified for the first time the cDNA sequences coding for a family of prothrombin activating enzymes from a total of six other Australian elapids including the coastal and inland taipan, red-bellied black, rough scaled, Stephen's banded and tiger snakes. Comparative analysis of these sequences has provided valuable information on the structure of these proteins that should aid in their development as a therapeutic agent. These include observed conservation of active site residues as well as structural domains between species, such as the sites of post-translational modifications including γ-carboxylation and N- and O-linked glycosylation. Given the known importance of these conserved domains, a chimeric molecule may be developed in the future (with adequate post-translational modifications) capable of acting as a tissue sealent in humans. Comparative phylogenetic and molecular analysis has also confirmed past functional classification of these toxins into group C and D prothrombin activators, which has previously been made on the basis of their cofactor requirement (Kini *et al.*, 2001a). This comparative analysis also extended to the cloning of factor V-like components of the group C prothrombin activators from the venom glands of the *Oxyuranus* species.

Steps were also taken to produce a recombinant form of the factor Xa-like prothrombin activator from the coastal taipan (*O. scutellatus*), which shares significant homology (92.1%) to that of the *P. textilis* sequence. It was hoped that this recombinant protein could be studied in functional assays to further delineate the various activities/roles of this molecule as well as act as a stepping block in the large scale production of protein for clinical trial as a haemostatic tissue sealant. Although recombinant protein was successfully expressed and purified, it demonstrated reduced activity compared to the native form from the common brown snake. Two key issues may be identified in the attempt to obtain an active protease via the recombinant expression of a factor Xa-like prothrombin activator. Firstly, correct cleavage and folding of the protein into a two-chain molecule with a light and heavy chain linked by a single disulfide bond is crucial. This is supported by the evidence that, although recombinant protein originally demonstrated no activity in a chromogenic assay, it was possible to activate the protein via treatment with the venom of a Russell's viper (RVV), which contains an activator of factor X. RVV subsequently cleaved the activation peptide that still remained bound to the light chain of the recombinant protein, where upon activity was observed. This concurs well with the observations of the protease detected directly by immunoblotting native venom, which appears as two separate chains (similar to that of the mammalian prothrombin activator) under reducing conditions. The second issue identified in the production of a recombinant protein that is functionally active within an *in vivo* environment is the necessity for appropriate post-translational modifications, particularly γ-carboxylation. This post-translational modification occurs at eleven conserved sites within the snake protease, and previous studies of its mammalian counterpart have shown that these residues are conserved and are important for the binding of calcium and hence the activity of the protein (Brown *et al.*, 2002; Morita and Jackson, 1986). Evidence provided by immunoblotting in this study, as well as previous recombinant protein expression studies using a similar expression system/cell line indicate that the recombinant *O. scutellatus* protease did not contain this post-translational modification, and hence would be expected to have a reduced activity in an *in vivo* environment (Roth *et al.*, 1993).

Detailed comparative analysis at the molecular level of factor Xa-like proteases from a select group of Australian snakes reveals a remarkable degree of conservation, consistent with their homologous effects upon the mammalian blood coagulation cascade (Kini *et al.*, 2001b). To further develop a group C protease as a therapeutic tissue sealant, a number of key points must first be addressed. The first of these is the selection of a protein expression system

capable of readily producing large quantities of recombinant protein with adequate post-translational modifications for activity within an *in vivo* environment. The second is the production of a construct that is correctly processed and folded into an active form, or the expression of truncated or chimeric form of the protein that demonstrates a similar or increased activity. To this end, the comparative molecular data provided in this study will prove highly valuable in the design of such a construct. The selected protein could then undergo a suite of functional studies including stability assays as well as a large-animal bleeding trial to determine its efficacy as a procoagulant tissue sealant.

Toxin cDNA Identification in Australian Snakes

An additional primary aim of this project was the further identification of known and novel toxin cDNA sequences from the venom glands of Australian elapids. Although the production and subsequent screening of a cDNA library from the venom gland of a snake has previously been performed (most notably from a viper, the Island jararaca, *Bothrops insularis*) this is the first study to employ microarray technology, to the extent of this author's knowledge, as a means of identifying venom gland specific genes from a snake (Junqueira-de-Azevedo Ide and Ho, 2002). Microarray production and screening has the distinct advantage in that by hybridising RNA from a tissue source other than that of a venom gland, it is possible to screen for transcripts that are venom gland specific, and hence more likely to be a constituent of the venom. This is clearly of benefit when only sequences corresponding to molecules that target mammalian systems, i.e. toxins, are of interest. As a consequence, multiple full-length toxin cDNA sequences were identified from the venom gland of the coastal taipan and subsequently cloned from related Australian elapids. This was made possible by the high degree of sequence relatedness between homologous genes in the different species, which even extended into the 3' and 5' untranslated regions. The most dominant toxin sequence identified from screens of the microarray was a pseudechetoxin-like clone, which belongs to the family of cysteine rich venom proteins. Although pseudechetoxin from the mulga, and its homolog pseudecin from the red-bellied black snake have previously been identified, this study represents the first description of these sequences in the other Australian snakes (with the exception of the rough-scaled snake where it was not detected) (Yamazaki *et al.*, 2002). These toxins are known to bind and block cyclic-nucleotide gated (CNG) ion channels, which have been identified in a number of body tissues including the brain, heart and kidneys and play a central role in signal transduction in retinal photoreceptors

and olfactory neurons (Brown *et al.*, 2003; Bradley *et al.*, 1997). The comparative analysis of this toxin detailed here will provide valuable information for further probing the structure and function of CNG ion channels.

Aside from the pseudechetoxin-like gene, multiple other toxins were identified from the venom gland of the coastal taipan. These included short and long chain α-neurotoxins, a myriad of phospholipase A_2 isoforms, a venom natriuretic peptide and a venom nerve growth factor-like transcript. All of these sequences determined here represent their first description at the nucleotide level and in many instances at the protein level. Similarly, multiple non-toxin genes were also isolated, most notably a calglandulin-like protein and a number of ribosomal sequences, which also represent a significant contribution to our understanding of the genetics of Australian elapid snakes. Indeed a number of unknown sequences were also cloned that demonstrated no identity upon database searches and therefore represent good leads for the identification of as yet uncharacterised, novel proteins.

Again, given the highly related nature of the Australian elapid snake family it was possible to identify homologous genes in other members of this group. The comparative analysis of phospholipase A_2 enzymes identified multiple proteins in many of the snakes. Given that this family of toxins may exert a wide range of varying physiological functions including digestive, haemorrhagic, myotoxic, haemolytic, hypotensive, oedema forming, platelet aggregating, convulsant, cardiotoxic and pre- and post-synaptic neurotoxic activities upon what appears to be very minor changes in primary structure, the multiple PLA_2 isoforms identified in this study provide an excellent platform for further investigating the relationship between structure and function within this family of molecules (Kini, 2003; Arni and Ward, 1996). That is, through expression of different isoforms and potential mutagenic studies it may be possible to identify key residues or structural properties that are important for a particular function (for example anti-coagulation) and hence make it possible to tailor therapeutic molecules that specifically exert these effects. It is also possible to associate already known proteins that were identified in this study with their function, for instance the mitogenic effects of the β-chain of taipoxin, to also delineate these relationships. Hence the reverse approach of identifying the activity of fractions or specific components purified directly from the venom will also play an important role in identifying the function of these molecules and will bolster the data obtained at the genetic level in this thesis.

Such an approach for studying the structural and functional relationships of toxin families is not only true of the PLA$_2$s, but may also be applied to the α-neurotoxins and venom natriuretic peptides. A total of ten short chain and seven long chain neurotoxins were cloned and identified in this study. Many of these proteins had a number of interesting structural features, for example the absence of conserved disulfide bonds in a number of long chain neurotoxins or amino acid substitutions at key functional sites. These changes may have significant implications for the neurotoxic properties of the venom of some of the world's most toxic snakes. A number of completely novel neurotoxins were also identified, one of which from the common brown snake was successfully expressed in a bacterial recombinant protein expression system and shown to be active in a functional assay. Hence this system may serve as a noteworthy platform for further characterisation of these proteins and their effect on the neuromuscular system of humans, again with the development of novel therapeutic or diagnostic agents in mind.

The same recombinant protein expression system was also used to produce functionally active isoforms of venom natriuretic peptides from the mulga and common brown snake. These are a relatively new class of venom proteins that exert a vasoactive effect in mammalian systems (Fry *et al.*, 2005; Stoupakis and Klapholz, 2003). This study demonstrates for the first time a direct, dose-dependent inhibition of angiotensin converting enzyme (ACE) by venom natriuretic peptides isolated from Australian elapid snakes. ACE is a key enzyme in the renin-angiotensin system and its inhibition would be predicted to result in, amongst other effects, vasorelaxation within a mammalian system (Lumbers, 1999). Indeed ACE inhibitors are currently being investigated as antihypertensive therapeutic agents (Toto *et al.*, 2004). The multiple unique natriuretic peptide isoforms identified for the first time from Australian elapids in this study vary considerably at the primary amino acid level. These clones may again be used to examine the structural relationship between natriuretic peptides and their vasoactive properties. Given their relatively small size, variation in amino acid composition and ease of purification in a recombinant system, they serve as ideal molecules for the identification of novel activities that may be of pharmacological importance. The role of these toxins in the venom is still not fully understood, although it is hypothesised that their vasorelaxative properties aid in the systemic delivery of other toxins.

Toxin Evolution and Variation

One of the common features of many of the toxin families identified in this study was their presence as multiple isoforms within the venom of any one snake. Toxin gene sequences may be subjected to frequent duplication events within the snake genome, often followed by functional and structural diversification at an accelerated rate, as evidenced by relatively conserved intronic sequences compared to gene exons (Kordis and Gubensek, 2000; Moura-da-Silva *et al.*, 1995). Hence these sequences often exist as large multigene families. It has been proposed that the presence of multiple isoforms of any one toxin has been driven by the necessity for the snake to immobilise, kill and digest a wide variety of prey sources depending on location, each containing its own specific molecular target site (Jeyaseelan *et al.*, 2000). This necessity also drives the accelerated rates of evolution observed in toxin gene sequences, as the snake strives to adapt to and overcome the development of further resistance within prey (Kini and Chan, 1999). By maintaining a basic structural framework, the snake has been able to adopt a multitude of various pharmacological effects just by altering a small number of key amino acids. This is particularly evident in the three-finger snake toxin family as well as the phospholipase A_2 enzymes, which demonstrate structural homology but a diversity of function (Fry *et al.*, 2003). For example although the short chain neurotoxin identified from the coastal taipan, Os SNTX-1, in this and other studies only weakly binds and inhibits nicotinic acetylcholine receptors in a mammalian system, it may have a significantly different effect at the amphibian neuromuscular junction (Zamudio *et al.*, 1996). Hence the comparative molecular data provided here will prove an invaluable tool for the identification of those changes that exert these various pharmacological effects with wide ranging implications not only for toxin function but the study of those mammalian systems that these toxins specifically target.

Multiple isoforms were observed in the eight Australian elapid neurotoxin, phospholipase A_2 and venom natriuretic peptide toxin families identified in this study. A notable exception however was the factor X- and factor V-like proteins, where only a single clone was identified in all snakes when present. This may be because such a large protein is metabolically expensive to produce, or possibly because its specific target within the coagulation cascade, prothrombin, is highly conserved in all prey species and hence only one isoform is required. It is also possible however that the prothrombin activator component is only recently acquired on an evolutionary scale and has not yet had time to undergo duplication and divergence

events. Hence there is a strong case for the cloning of the full genomic gene sequence of this and other toxin sequences from Australian elapids. As with cDNA sequences, there has been little research into the genomic DNA sequences of toxins from Australian elapids. The most comprehensive study to date has been the identification of a neurotoxin multigene family from the common brown snake, but otherwise there has been little research focus on the toxin genes from the eight snakes investigated in this study (Gong *et al.*, 2000). Genomic studies also have the added advantage in that DNA isolation does not require sacrificing an animal: a blood sample is adequate. Such comparative genetic studies will not only shed light on the rate of evolution of Australian snake toxin genes but also the origin of these gene sequences. The cDNA clones identified in this study would again form a solid foundation for the basis of such a study.

The nucleotide data described for the first time in this study is also an important contribution to our understanding of the phylogenetic relationship of Australian elapids. Previous classification of elapid snakes has been on the basis of a number of parameters including internal and external morphology, immunological distances and ecological and biochemical means (Hutchinson, 1990). Molecular sequence data can prove to be a powerful tool in the inference of gene trees from species trees in that not only can it indicate the order of divergence between species but also give a measure of the timing of that divergence (Wuster *et al.*, 2005; Keogh *et al.*, 1998; Slowinski *et al.*, 1997). The phylogenetic data described in this study supports association of the inland and coastal taipan into one genus, *Oxyuranus*, as well as the red-bellied black snake and mulga into a single genus, *Pseudechis*. It also supports the close relationship between the *Oxyuranus* species and the common brown snake *Pseudonaja textilis* described by Keogh *et al.* (1998). There appears to be a significant association between the *Tropidechis* and *Notechis* species with *Hoplocephalus stephensii* more distantly related for pseudechetoxin-like proteins, the short chain neurotoxins and the calglandulin-like transcripts, however this relationship is less well defined for the factor X-like protease. The bootstrap values described however for this last association do not strongly support evidence for a closer link between *N. scutatus* and *H. stephensii* as opposed to the traditional *N. scutatus – T. carinatus* association. Hence the genetic data for the various toxins described in this thesis are a valuable contribution to further understanding the phylogenetic relationship of Australian elapid snakes.

Future Directions and Implications of This Study

Ultimately, the next step in our understanding the function of toxins and their potential applications is to determine the biochemical, physiological and structural properties of individual components. As alluded to earlier, this may be tackled via a two-pronged approach. Firstly characterisation of the properties of the crude and fractionated venom in conjunction with proteomic analysis will provide information on not only the different activities within the venom of single snake species, but also the family of toxins responsible for this activity. A proteomic approach also has the advantage in that all unknown peptides are at least known to exist in the venom, whereas this is not always the case of novel genetic sequences. Secondly, the genomic approach described in this thesis allows for the exact identification of multiple isoforms of any given toxin family, and through recombinant protein expression and mutagenic studies it will be possible to exactly define the function of each of these molecules or parts thereof. Only then will it be possible to specifically tailor these molecules for therapeutic or diagnostic applications. It is important to keep in mind that this study has only focussed on the toxic components of the coastal taipan, with subsequent identification of related proteins in other species. Proteins unique to the venom of any of these other snakes will not be identified by screens of an *O. scutellatus* cDNA library and therefore may go undiscovered. Therefore a comparative proteomic approach is again important for identification of all components within the venom of a particular snake. Conversely it may become easier to study the entire transcriptome of any snake venom gland with advances in DNA technologies, including recent evidence that it is possible to isolate RNA directly from the venom, negating the need to euthanise the animal, which are often rare, expensive or protected under government legislation (Chen *et al.*, 2002). In this manner it would be possible to vastly expand the number of available snake cDNA samples, making it possible to classify other functionally different isoforms of known proteins, or discover completely novel families. Nonetheless, this study has already successfully identified multiple unique toxin families within the coastal taipan and other related Australian snakes at the genetic level with major contributions to our understanding of the structural components of these venoms.

In summary, this thesis has described the cloning, partial characterisation and comparative analysis of numerous toxin gene sequences isolated from a cohort of Australian elapids. It represents the most comprehensive study to date of the toxic components of the venom of

these snakes at a molecular level. The implications of the findings of this project are wide ranging, providing valuable information for further understanding the toxicology of Australian snake venoms as well as the development of potential pharmacological agents from its components. Collectively, this work provides a basis for the isolation of the entire plethora of pharmacologically active components in Australian snake venoms as well as providing specific examples of how these components may be further characterised on a functional level.

References

Adachi, J. and Hasegawa, M. (1996) MOLPHY version 2.3: program for molecular phylogenetics based on maximum likelihood. *Comput Sci Monogr*. **28**: 1-150.

Afifiyan, F., Armugam, A., Tan, C.H., Gopalakrishnakone, P. and Jeyaseelan, K. (1999) Postsynaptic alpha-neurotoxin gene of the spitting cobra, *Naja naja sputatrix*: structure, organization, and phylogenetic analysis. *Genome Res*. **9**: 259-266.

Amininasab, M., Elmi, M.M., Endlich, N., Endlich, K., Parekh, N., Naderi-Manesh, H., *et al* (2004) Functional and structural characterization of a novel member of the natriuretic family of peptides from the venom of *Pseudocerastes persicus*. *FEBS Lett*. **557**: 104-108.

Antil, S., Servent, D. and Menez, A. (1999) Variability among the sites by which curaremimetic toxins bind to torpedo acetylcholine receptor, as revealed by identification of the functional residues of alpha-cobratoxin. *J Biol Chem*. **274**: 34851-34858.

Antil-Delbeke, S., Gaillard, C., Tamiya, T., Corringer, P.J., Changeux, J.P., Servent, D. and Menez, A. (2000) Molecular determinants by which a long chain toxin from snake venom interacts with the neuronal alpha 7-nicotinic acetylcholine receptor. *J Biol Chem*. **275**: 29594-29601.

Arni, R.K. and Ward, R.J. (1996) Phospholipase A2--a structural review. *Toxicon*. **34**: 827-841.

Arthur, C.K., McCallum, D., Loveday, D.J., Collins, A., Isbister, J.P. and Fisher, M.M. (1991) Effects of taipan (*Oxyuranus scutellatus*) venom on erythrocyte morphology and blood viscosity in a human victim in vivo and in vitro. *Trans R Soc Trop Med Hyg*. **85**: 401-403.

Barchan, D., Ovadia, M., Kochva, E. and Fuchs, S. (1995) The binding site of the nicotinic acetylcholine receptor in animal species resistant to alpha-bungarotoxin. *Biochemistry*. **34**: 9172-9176.

Barchan, D., Kachalsky, S., Neumann, D., Vogel, Z., Ovadia, M., Kochva, E. and Fuchs, S. (1992) How the mongoose can fight the snake: the binding site of the mongoose acetylcholine receptor. *Proc Natl Acad Sci U S A*. **89**: 7717-7721.

Baugh, R.J. and Krishnaswamy, S. (1996) Role of the activation peptide domain in human factor X activation by the extrinsic Xase complex. *J Biol Chem*. **271**: 16126-16134.

Bell, K.L., Sutherland, S.K. and Hodgson, W.C. (1998) Some pharmacological studies of venom from the inland taipan (*Oxyuranus microlepidotus*). *Toxicon*. **36**: 63-74.

Best, P.J., Burnett, J.C., Wilson, S.H., Holmes, D.R., Jr. and Lerman, A. (2002) Dendroaspis natriuretic peptide relaxes isolated human arteries and veins. *Cardiovasc Res*. **55**: 375-384.

Bouet, F., Menez, A., Hider, R.C. and Fromageot, P. (1982) Separation of intermediates in the refolding of reduced erabutoxin b by analytical isoelectric focusing in layers of polyacrylamide gel. *Biochem J*. **201**: 495-499.

Bradley, J., Zhang, Y., Bakin, R., Lester, H.A., Ronnett, G.V. and Zinn, K. (1997) Functional expression of the heteromeric "olfactory" cyclic nucleotide-gated channel in the hippocampus: a potential effector of synaptic plasticity in brain neurons. *J Neurosci*. **17**: 1993-2005.

Braud, S., Bon, C. and Wisner, A. (2000) Snake venom proteins acting on hemostasis. *Biochimie*. **82**: 851-859.

Broad, A.J., Sutherland, S.K. and Coulter, A.R. (1979) The lethality in mice of dangerous Australian and other snake venom. *Toxicon*. **17**: 661-664.

Brown, A.M., Yatani, A., Lacerda, A.E., Gurrola, G.B. and Possani, L.D. (1987) Neurotoxins that act selectively on voltage-dependent cardiac calcium channels. *Circ Res*. **61**: I6-9.

Brown, M.A., Hambe, B., Furie, B., Furie, B.C., Stenflo, J. and Stenberg, L.M. (2002) Detection of vitamin K-dependent proteins in venoms with a monoclonal antibody specific for gamma-carboxyglutamic acid. *Toxicon*. **40**: 447-453.

Brown, R.L., Haley, T.L., West, K.A. and Crabb, J.W. (1999) Pseudechetoxin: a peptide blocker of cyclic nucleotide-gated ion channels. *Proc Natl Acad Sci U S A*. **96**: 754-759.

Brown, R.L., Lynch, L.L., Haley, T.L. and Arsanjani, R. (2003) Pseudechetoxin binds to the pore turret of cyclic nucleotide-gated ion channels. *J Gen Physiol*. **122**: 749-760.

Burns, M.E. and Baylor, D.A. (2001) Activation, deactivation, and adaptation in vertebrate photoreceptor cells. *Annu Rev Neurosci*. **24**: 779-805.

Buttery, J.E. and Stuart, S. (1993) Assessment and optimization of kinetic methods for angiotensin-converting enzyme in plasma. *Clin Chem*. **39**: 312-316.

Chang, L.S., Wu, P.F., Liou, J.C., Chiang-Lin, W.H. and Yang, C.C. (2004) Chemical modification of arginine residues of *Notechis scutatus scutatus* notexin. *Toxicon*. **44**: 491-497.

Chattopadhyay, A., James, H.L. and Fair, D.S. (1992) Molecular recognition sites on factor Xa which participate in the prothrombinase complex. *J Biol Chem*. **267**: 12323-12329.

Chen, H.H. and Burnett, J.C., Jr. (1999) The natriuretic peptides in heart failure: diagnostic and therapeutic potentials. *Proc Assoc Am Physicians*. **111**: 406-416.

Chen, H.H. and Burnett, J.C. (2000) Natriuretic peptides in the pathophysiology of congestive heart failure. *Curr Cardiol Rep*. **2**: 198-205.

Chen, S., Guo, J.H., Saiyin, H., Chen, L., Zhou, G.J., Huang, C.Q. and Yu, L. (2004) Cloning and characterization of human CAGLP gene encoding a novel EF-hand protein. *DNA Seq*. **15**: 365-368.

Chen, T., Bjourson, A.J., Orr, D.F., Kwok, H., Rao, P., Ivanyi, C. and Shaw, C. (2002) Unmasking venom gland transcriptomes in reptile venoms. *Anal Biochem*. **311**: 152-156.

Chester, A. and Crawford, G.P. (1982) In vitro coagulant properties of venoms from Australian snakes. *Toxicon*. **20**: 501-504.

Chin, D. and Means, A.R. (2000) Calmodulin: a prototypical calcium sensor. *Trends Cell Biol*. **10**: 322-328.

Cho, Y., Somer, B.G. and Amatya, A. (1999) Natriuretic peptides and their therapeutic potential. *Heart Dis*. **1**: 305-328.

Colquhoun, L.M. and Patrick, J.W. (1997) Pharmacology of neuronal nicotinic acetylcholine receptor subtypes. *Adv Pharmacol*. **39**: 191-220.

Colucci, W.S., Elkayam, U., Horton, D.P., Abraham, W.T., Bourge, R.C., Johnson, A.D*., et al* (2000) Intravenous nesiritide, a natriuretic peptide, in the treatment of decompensated congestive heart failure. Nesiritide Study Group. *N Engl J Med*. **343**: 246-253.

Crachi, M.T., Hammer, L.W. and Hodgson, W.C. (1999) The effects of antivenom on the in vitro neurotoxicity of venoms from the taipans *Oxyuranus scutellatus*, *Oxyuranus microlepidotus* and *Oxyuranus scutellatus canni*. *Toxicon*. **37**: 1771-1778.

Daltry, J.C., Wuster, W. and Thorpe, R.S. (1996) Diet and snake venom evolution. *Nature*. **379**: 537-540.

Dambisya, Y.M., Lee, T.L. and Gopalakrishnakone, P. (1995) Anticoagulant effects of Pseudechis australis (Australian king brown snake) venom on human blood: a computerized thromboelastography study. *Toxicon*. **33**: 1378-1382.

Danse, J.M. and Garnier, J.M. (1993) Molecular cloning of a cDNA encoding a nerve growth factor precursor from the krait, *Bungarus multicinctus*. *Growth Factors*. **8**: 77-86.

Davie, E.W., Fujikawa, K. and Kisiel, W. (1991) The coagulation cascade: initiation, maintenance, and regulation. *Biochemistry*. **30**: 10363-10370.

de Weille, J.R., Schweitz, H., Maes, P., Tartar, A. and Lazdunski, M. (1991) Calciseptine, a peptide isolated from black mamba venom, is a specific blocker of the L-type calcium channel. *Proc Natl Acad Sci U S A*. **88**: 2437-2440.

Di Scipio, R.G., Hermodson, M.A., Yates, S.G. and Davie, E.W. (1977) A comparison of human prothrombin, factor IX (Christmas factor), factor X (Stuart factor), and protein S. *Biochemistry*. **16**: 698-706.

Doorty, K.B., Bevan, S., Wadsworth, J.D. and Strong, P.N. (1997) A novel small conductance Ca2+-activated K+ channel blocker from *Oxyuranus scutellatus* taipan venom. Re-evaluation of taicatoxin as a selective Ca2+ channel probe. *J Biol Chem*. **272**: 19925-19930.

Du, X.Y. and Clemetson, K.J. (2002) Snake venom L-amino acid oxidases. *Toxicon*. **40**: 659-665.

Eaker, D. and Porath, J. (1967) The amino acid sequence of a neurotoxin from *Naja nigricollis* venom. *Jpn J Microbiol*. **11**: 353-355.

Evans, T.C., Jr., Benner, J. and Xu, M.Q. (1999) The cyclization and polymerization of bacterially expressed proteins using modified self-splicing inteins. *J Biol Chem*. **274**: 18359-18363.

Faiz, M.A., Falkous, G., Harris, J.B. and Mantle, D. (1996) Comparison of protease and related enzyme activities in snake venoms. *Comp Biochem Physiol B Biochem Mol Biol*. **113**: 199-204.

Fatehi, M., Rowan, E.G., Harvey, A.L. and Harris, J.B. (1994) The effects of five phospholipases A2 from the venom of king brown snake, *Pseudechis australis*, on nerve and muscle. *Toxicon*. **32**: 1559-1572.

Filippovich, I., Sorokina, N., Masci, P.P., de Jersey, J., Whitaker, A.N., Winzor, D.J.*, et al* (2002) A family of textilinin genes, two of which encode proteins with antihaemorrhagic properties. *Br J Haematol*. **119**: 376-384.

Filippovich, I., Sorokina, N., St Pierre, L., Flight, S., de Jersey, J., Perry, N.*, et al* (2005) Cloning and functional expression of venom prothrombin activator protease from *Pseudonaja textilis* with whole blood procoagulant activity. *Br J Haematol*. **131**: 237-246.

Fletcher, J.E. and Jiang, M.S. (1995) Presynaptically acting snake venom phospholipase A2 enzymes attack unique substrates. *Toxicon*. **33**: 1565-1576.

Fohlman, J. (1979) Comparison of two highly toxic Australian snake venoms: the taipan (Oxyuranus S. scutellatus) and the fierce snake (*Parademansia microlepidotus*). *Toxicon*. **17**: 170-172.

Fohlman, J., Eaker, D., Karlsoon, E. and Thesleff, S. (1976) Taipoxin, an extremely potent presynaptic neurotoxin from the venom of the australian snake taipan (*Oxyuranus s. scutellatus*). Isolation, characterization, quaternary structure and pharmacological properties. *Eur J Biochem*. **68**: 457-469.

Fontana, F. (1781) *Treatise of the venom of the viper*. London.

Francis, B., John, T.R., Seebart, C. and Kaiser, II (1991) New toxins from the venom of the common tiger snake (*Notechis scutatus scutatus*). *Toxicon*. **29**: 85-96.

Francis, B., Williams, E.S., Seebart, C. and Kaiser, II (1993) Proteins isolated from the venom of the common tiger snake (*Notechis scutatus scutatus*) promote hypotension and hemorrhage. *Toxicon*. **31**: 447-458.

Francis, B., Coffield, J.A., Simpson, L.L. and Kaiser, II (1995) Amino acid sequence of a new type of toxic phospholipase A2 from the venom of the Australian tiger snake (*Notechis scutatus scutatus*). *Arch Biochem Biophys*. **318**: 481-488.

Fry, B.G. (1999) Structure-function properties of venom components from Australian elapids. *Toxicon*. **37**: 11-32.

Fry, B.G. and Wuster, W. (2004) Assembling an arsenal: origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences. *Mol Biol Evol*. **21**: 870-883.

Fry, B.G., Wuster, W., Kini, R.M., Brusic, V., Khan, A., Venkataraman, D. and Rooney, A.P. (2003) Molecular evolution and phylogeny of elapid snake venom three-finger toxins. *J Mol Evol*. **57**: 110-129.

Fry, B.G., Wickramaratana, J.C., Lemme, S., Beuve, A., Garbers, D., Hodgson, W.C. and Alewood, P. (2005) Novel natriuretic peptides from the venom of the inland taipan (*Oxyuranus microlepidotus*): isolation, chemical and biological characterisation. *Biochem Biophys Res Commun*. **327**: 1011-1015.

Geh, S.L., Rowan, E.G. and Harvey, A.L. (1992) Neuromuscular effects of four phospholipases A2 from the venom of *Pseudechis australis*, the Australian king brown snake. *Toxicon*. **30**: 1051-1057.

Golovanov, A.P., Lomize, A.L., Arseniev, A.S., Utkin, Y.N. and Tsetlin, V.I. (1993) Two-dimensional 1H-NMR study of the spatial structure of neurotoxin II from *Naja naja oxiana*. *Eur J Biochem*. **213**: 1213-1223.

Gong, N., Armugam, A. and Jeyaseelan, K. (1999) Postsynaptic short-chain neurotoxins from *Pseudonaja textilis*. cDNA cloning, expression and protein characterization. *European Journal of Biochemistry*. **265**: 982-989.

Gong, N., Armugam, A. and Jeyaseelan, K. (2000) Molecular cloning, characterization and evolution of the gene encoding a new group of short-chain alpha-neurotoxins in an Australian elapid, *Pseudonaja textilis*. *FEBS Letters*. **473**: 303-310.

Gong, N., Armugam, A., Mirtschin, P. and Jeyaseelan, K. (2001) Cloning and characterization of the pseudonajatoxin b precursor. *Biochem J*. **358**: 647-656.

Grant, G.A. and Chiappinelli, V.A. (1985) kappa-Bungarotoxin: complete amino acid sequence of a neuronal nicotinic receptor probe. *Biochemistry*. **24**: 1532-1537.

Grant, G.A., Al-Rabiee, R., Xu, X.L. and Zhang, Y. (1997) Critical interactions at the dimer interface of kappa-bungarotoxin, a neuronal nicotinic acetylcholine receptor antagonist. *Biochemistry*. **36**: 3353-3358.

Grishin, E.V., Sukhikh, A.P., Slobodyan, L.N., Ovchinnikov Yu, A. and Sorokin, V.M. (1974) Amino acid sequence of neurotoxin I from *Naja naja oxiana* venom. *FEBS Lett*. **45**: 118-121.

Hains, P.G., Nield, B., Sekuloski, S., Dunn, R. and Broady, K. (2001) Sequencing and two-dimensional structure prediction of a phospholipase A(2) inhibitor from the serum of the common tiger snake (*Notechis scutatus*). *J Mol Biol*. **312**: 875-884.

Halpert, J. and Eaker, D. (1975) Amino acid sequence of a presynaptic neurotoxin from the venom of *Notechis scutatus scutatus* (Australian tiger snake). *J Biol Chem*. **250**: 6990-6997.

Halpert, J., Fohlman, J. and Eaker, D. (1979) Amino acid sequence of a postsynaptic neurotoxin from the venom of the Australian tiger snake *Notechis scutatus scutatus*. *Biochimie*. **61**: 719-723.

Han, B. and Hasin, Y. (2003) Cardiovascular effects of natriuretic peptides and their interrelation with endothelin-1. *Cardiovasc Drugs Ther*. **17**: 41-52.

Harris, J.B. (1997) Toxic phospholipases in snake venom: an introductory review. In *Venomous Snakes: Ecology, Evolution and Snakebite*. Thorpe, R.S., Wuster, W., and Malhorta, A. (eds.) London: Zoological Society of London, pp. 235-250.

Harris, J.B. and Maltin, C.A. (1982) Myotoxic activity of the crude venom and the principal neurotoxin, taipoxin, of the Australian taipan, *Oxyuranus scutellatus*. *Br J Pharmacol*. **76**: 61-75.

Harris, J.B., Karlsson, E. and Thesleff, S. (1973) Effects of an isolated toxin from Australian tiger snake (*Notechis scutatus scutatus*) venom at the mammalian neuromuscular junction. *Br J Pharmacol*. **47**: 141-146.

Harris, J.B., Vater, R., Wilson, M. and Cullen, M.J. (2003) Muscle fibre breakdown in venom-induced muscle degeneration. *J Anat*. **202**: 363-372.

Harvey, A.L., Bradley, K.N., Cochran, S.A., Rowan, E.G., Pratt, J.A., Quillfeldt, J.A. and Jerusalinsky, D.A. (1998) What can toxins tell us for drug discovery? *Toxicon*. **36**: 1635-1640.

He, X., Chow, D., Martick, M.M. and Garcia, K.C. (2001) Allosteric activation of a spring-loaded natriuretic peptide receptor dimer by hormone. *Science*. **293**: 1657-1662.

Heise, P.J., Maxson, L.R., Dowling, H.G. and Hedges, S.B. (1995) Higher-level snake phylogeny inferred from mitochondrial DNA sequences of 12S rRNA and 16S rRNA genes. *Mol Biol Evol*. **12**: 259-265.

Hogg, R.C., Trequattrini, C., Catacuzzeno, L., Petris, A., Franciolini, F. and Adams, D.J. (1999) Mechanisms of verapamil inhibition of action potential firing in rat intracardiac ganglion neurons. *J Pharmacol Exp Ther*. **289**: 1502-1508.

Holmquist, B., Bunning, P. and Riordan, J.F. (1979) A continuous spectrophotometric assay for angiotensin converting enzyme. *Anal Biochem*. **95**: 540-548.

Hori, H. and Tamiya, N. (1976) Preparation and activity of guanidinated or acetylated erabutoxins. *Biochem J*. **153**: 217-222.

Hutchinson, M.N. (1990) The generic classification of the Australian terrestrial elapid snakes. *Mem. Queensland Mus*. **28**: 397-405.

Jackson, C.M. (1984) Factor X. *Prog Hemost Thromb*. **7**: 55-109.

Jackson, C.M. and Nemerson, Y. (1980) Blood coagulation. *Annu Rev Biochem*. **49**: 765-811.

Jackson, M.R. (2001) Fibrin sealants in surgical practice: An overview. *Am J Surg*. **182**: 1S-7S.

James, H.L. (1994) Physiology and biochemistry of factor X. In *Haemostasis and Thrombosis*. Bloom, A.L., Forbes, C.D., Thomas, D.P., and Tuddenham, E.G.D. (eds.) Singapore: Longman Singapore Publishers (Pte) Ltd, pp. 439-.

Jerusalinsky, D. and Harvey, A.L. (1994) Toxins from mamba venoms: small proteins with selectivities for different subtypes of muscarinic acetylcholine receptors. *Trends Pharmacol Sci*. **15**: 424-430.

Jeyaseelan, K., Armugam, A., Donghui, M. and Tan, N.H. (2000) Structure and phylogeny of the venom group I phospholipase A(2) gene. *Mol Biol Evol*. **17**: 1010-1021.

Jin, Y., Lu, Q., Zhou, X., Zhu, S., Li, R., Wang, W. and Xiong, Y. (2003) Purification and cloning of cysteine-rich proteins from *Trimeresurus jerdonii* and *Naja atra* venoms. *Toxicon*. **42**: 539-547.

Joseph, J.S., Chung, M.C., Jeyaseelan, K. and Kini, R.M. (1999) Amino acid sequence of trocarin, a prothrombin activator from *Tropidechis carinatus* venom: its structural similarity to coagulation factor Xa. *Blood*. **94**: 621-631.

Joseph, J.S., Valiyaveettil, M., Gowda, D.C. and Kini, R.M. (2003) Occurrence of O-linked Xyl-GlcNAc and Xyl-Glc disaccharides in trocarin, a factor Xa homolog from snake venom. *J Thromb Haemost*. **1**: 545-550.

Junqueira-de-Azevedo, I.L., Farsky, S.H., Oliveira, M.L. and Ho, P.L. (2001) Molecular cloning and expression of a functional snake venom vascular endothelium growth factor (VEGF) from the *Bothrops insularis* pit viper. A new member of the VEGF family of proteins. *J Biol Chem*. **276**: 39836-39842.

Junqueira-de-Azevedo Ide, L. and Ho, P.L. (2002) A survey of gene expression and diversity in the venom glands of the pitviper snake *Bothrops insularis* through the generation of expressed sequence tags (ESTs). *Gene*. **299**: 279-291.

Junqueira-de-Azevedo Ide, L., Pertinhez, T., Spisni, A., Carreno, F.R., Farah, C.S. and Ho, P.L. (2003) Cloning and expression of calglandulin, a new EF-hand protein from the venom glands of *Bothrops insularis* snake in E. coli. *Biochim Biophys Acta*. **1648**: 90-98.

Kadkhodayan, S., Knapp, M.S., Schmidt, J.J., Fabes, S.E., Rupp, B. and Balhorn, R. (2000) Cloning, expression, and one-step purification of the minimal essential domain of the light chain of botulinum neurotoxin type A. *Protein Expr Purif*. **19**: 125-130.

Kalafatis, M. and Beck, D.O. (2002) Identification of a binding site for blood coagulation factor Xa on the heavy chain of factor Va. Amino acid residues 323-331 of factor V represent an interactive site for activated factor X. *Biochemistry*. **41**: 12715-12728.

Kalafatis, M., Rand, M.D. and Mann, K.G. (1994) The mechanism of inactivation of human factor V and human factor Va by activated protein C. *J Biol Chem*. **269**: 31869-31880.

Kashima, S., Soares, A.M., Roberto, P.G., Pereira, J.O., Astolfi-Filho, S., Cintra, A.O.*, et al* (2002) cDNA sequence and molecular modeling of a nerve growth factor from *Bothrops jararacussu* venomous gland. *Biochimie*. **84**: 675-680.

Keogh, J.S., Shine, R. and Donnellan, S. (1998) Phylogenetic relationships of terrestrial Australo-Papuan elapid snakes (subfamily *Hydrophiinae*) based on cytochrome b and 16S rRNA sequences. *Mol Phylogenet Evol*. **10**: 67-81.

Kim, H.S. and Tamiya, N. (1982) Amino acid sequences of two novel long-chain neurotoxins from the venom of the sea snake *Laticauda colubrina*. *Biochem J*. **207**: 215-223.

Kini, R.M. (2002) Molecular moulds with multiple missions: functional sites in three-finger toxins. *Clin Exp Pharmacol Physiol*. **29**: 815-822.

Kini, R.M. (2003) Excitement ahead: structure, function and mechanism of snake venom phospholipase A2 enzymes. *Toxicon*. **42**: 827-840.

Kini, R.M. and Chan, Y.M. (1999) Accelerated evolution and molecular surface of venom phospholipase A2 enzymes. *J Mol Evol*. **48**: 125-132.

Kini, R.M., Morita, T. and Rosing, J. (2001a) Classification and nomenclature of prothrombin activators isolated from snake venoms. *Thrombosis & Haemostasis*. **86**: 710-711.

Kini, R.M., Rao, V.S. and Joseph, J.S. (2001b) Procoagulant proteins from snake venoms. *Haemostasis*. **31**: 218-224.

Kisiel, W., Hermodson, M.A. and Davie, E.W. (1976) Factor X activating enzyme from Russell's viper venom: isolation and characterization. *Biochemistry*. **15**: 4901-4906.

Koller, K.J., Lowe, D.G., Bennett, G.L., Minamino, N., Kangawa, K., Matsuo, H. and Goeddel, D.V. (1991) Selective activation of the B natriuretic peptide receptor by C-type natriuretic peptide (CNP). *Science*. **252**: 120-123.

Kordis, D. and Gubensek, F. (2000) Adaptive evolution of animal toxin multigene families. *Gene*. **261**: 43-52.

Kostiza, T. and Meier, J. (1996) Nerve growth factors from snake venoms: chemical properties, mode of action and biological significance. *Toxicon*. **34**: 787-806.

Kumar, S., Tamura, K., Jakobsen, I.B. and Nei, M. (2001) MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics*. **17**: 1244-1245.

Lambeau, G., Ancian, P., Nicolas, J.P., Beiboer, S.H., Moinier, D., Verheij, H. and Lazdunski, M. (1995) Structural elements of secretory phospholipases A2 involved in the binding to M-type receptors. *J Biol Chem*. **270**: 5534-5540.

Lewis, R.J. and Garcia, M.L. (2003) Therapeutic potential of venom peptides. *Nat Rev Drug Discov*. **2**: 790-802.

Li, M., Fry, B.G. and Kini, R.M. (2005) Eggs-Only Diet: Its Implications for the Toxin Profile Changes and Ecology of the Marbled Sea Snake (*Aipysurus eydouxii*). *J Mol Evol*. **60**: 81-89.

Lind, P. (1982) Amino-acid sequence of the beta 1 isosubunit of taipoxin, an extremely potent presynaptic neurotoxin from the Australian snake taipan (*Oxyuranus s. scutellatus*). *Eur J Biochem*. **128**: 71-75.

Lind, P. and Eaker, D. (1982) Amino-acid sequence of the alpha-subunit of taipoxin, an extremely potent presynaptic neurotoxin from the Australian snake taipan (*Oxyuranus s. scutellatus*). *Eur J Biochem*. **124**: 441-447.

Lipps, B.V. (2000) Isolation of subunits, alpha, beta and gamma of the complex taipoxin from the venom of Australian taipan snake (*Oxyuranus s. scutellatus*): characterization of beta taipoxin as a potent mitogen. *Toxicon*. **38**: 1845-1854.

Lisy, O., Jougasaki, M., Heublein, D.M., Schirger, J.A., Chen, H.H., Wennberg, P.W. and Burnett, J.C. (1999) Renal actions of synthetic dendroaspis natriuretic peptide. *Kidney Int*. **56**: 502-508.

Lumbers, E.R. (1999) Angiotensin and aldosterone. *Regul Pept*. **80**: 91-100.

Maack, T. (1992) Receptors of atrial natriuretic factor. *Annu Rev Physiol*. **54**: 11-27.

MacFarlane, R.G. and Barnett, B. (1934) The haemostatic posibilities of snake venom. *Lancet*. **ii**: 985-987.

MacGillivray, R.T. and Fung, M.R. (1989) Molecular biology of factor X. *Baillieres Clin Haematol*. **2**: 897-917.

MacHeroux, P., Seth, O., Bollschweiler, C., Schwarz, M., Kurfurst, M., Au, L.C. and Ghisla, S. (2001) L-amino-acid oxidase from the Malayan pit viper *Calloselasma rhodostoma*. Comparative sequence analysis and characterization of active and inactive forms of the enzyme. *Eur J Biochem*. **268**: 1679-1686.

Maeda, N. and Tamiya, N. (1978) Three neurotoxins from the venom of a sea snake *Astrotia stokesii*, including two long-chain neurotoxic proteins with amidated C-termini. *Biochem J*. **175**: 507-517.

Markland, F.S. (1998) Snake venoms and the hemostatic system. *Toxicon*. **36**: 1749-1800.

Marsh, N.A. (1994) Snake venoms affecting the haemostatic mechanism - a consideration of their mechanisms, practical applications and biological significance. *Blood Coagul Fibrinolysis*. **5**: 399-410.

Marsh, N.A., Fyffe, T.L. and Bennett, E.A. (1997) Isolation and partial characterization of a prothrombin-activating enzyme from the venom of the Australian rough-scaled snake (*Tropidechis carinatus*). *Toxicon*. **35**: 563-571.

Marshall, L.R. and Herrmann, R.P. (1983) Coagulant and anticoagulant actions of Australian snake venoms. *Thromb Haemost*. **50**: 707-711.

Masci, P.P., Whitaker, A.N. and de Jersey, J. (1988) Purification and characterization of a prothrombin activator from the venom of the Australian brown snake, *Pseudonaja textilis textilis*. *Biochem Int*. **17**: 825-835.

Masci, P.P., Whitaker, A.N., Sparrow, L.G., de Jersey, J., Winzor, D.J., Watters, D.J.*, et al* (2000) Textilinins from Pseudonaja textilis textilis. Characterization of two plasmin inhibitors that reduce bleeding in an animal model. *Blood Coagul Fibrinolysis*. **11**: 385-393.

Mashiko, H. and Takahashi, H. (2002) Cysteine proteinase inhibitors in elapid and hydrophiid snake venoms. *Toxicon*. **40**: 1275-1281.

Matsui, T., Fujimura, Y. and Titani, K. (2000) Snake venom proteases affecting hemostasis and thrombosis. *Biochim Biophys Acta*. **1477**: 146-156.

McDowell, S.B. (1970) On the status and relationship of the Solomon Island elapid snakes. *J. Zool. London*. **161**: 145-190.

McLane, M.A., Sanchez, E.E., Wong, A., Paquette-Straub, C. and Perez, J.C. (2004) Disintegrins. *Curr Drug Targets Cardiovasc Haematol Disord*. **4**: 327-355.

Mebs, D. (2001) Toxicity in animals. Trends in evolution? *Toxicon*. **39**: 87-96.

Mebs, D., Ehrenfeld, M. and Samejima, Y. (1983) Local necrotizing effect of snake venoms on skin and muscle: relationship to serum creatine kinase. *Toxicon*. **21**: 393-404.

Menez, A. (1998) Functional architectures of animal toxins: a clue to drug design? *Toxicon*. **36**: 1557-1572.

Menez, A., Bouet, F., Guschlbauer, W. and Fromageot, P. (1980) Refolding of reduced short neurotoxins: circular dichroism analysis. *Biochemistry*. **19**: 4166-4172.

Mengden, G.A. (1983) The taxonomy of Australian elapid snakes, a review. *Rec. Aus. Mus.* **35**: 195-222.

Messier, T.L., Pittman, D.D., Long, G.L., Kaufman, R.J. and Church, W.R. (1991) Cloning and expression in COS-1 cells of a full-length cDNA encoding human coagulation factor X. *Gene*. **99**: 291-294.

Meunier, F.A., Feng, Z.P., Molgo, J., Zamponi, G.W. and Schiavo, G. (2002) Glycerotoxin from Glycera convoluta stimulates neurosecretion by up-regulating N-type Ca^{2+} channel activity. *Embo J*. **21**: 6733-6743.

Mirtschin, P.J., Shine, R., Nias, T.J., Dunstan, N.L., Hough, B.J. and Mirtschin, M. (2002) Influences on venom yield in Australian tigersnakes (*Notechis scutatus*) and brownsnakes (*Pseudonaja textilis*: *Elapidae, Serpentes*). *Toxicon*. **40**: 1581-1592.

Misono, K.S. (2002) Natriuretic peptide receptor: structure and signaling. *Mol Cell Biochem*. **230**: 49-60.

Mollier, P., Brochier, G. and Morot Gaudry-Talarmain, Y. (1990) The action of notexin from tiger snake venom (*Notechis scutatus scutatus*) on acetylcholine release and compartmentation in synaptosomes from electric organ of Torpedo marmorata. *Toxicon*. **28**: 1039-1052.

Morita, T. (2004) C-type lectin-related proteins from snake venoms. *Curr Drug Targets Cardiovasc Haematol Disord*. **4**: 357-373.

Morita, T. and Iwanaga, S. (1978) Purification and properties of prothrombin activator from the venom of *Echis carinatus*. *J Biochem (Tokyo)*. **83**: 559-570.

Morita, T. and Jackson, C.M. (1986) Preparation and properties of derivatives of bovine factor X and factor Xa from which the gamma-carboxyglutamic acid containing domain has been removed. *J Biol Chem*. **261**: 4015-4023.

Morrison, J.J., Pearn, J.H., Charles, N.T. and Coulter, A.R. (1983) Further studies on the mass of venom injected by Elapid snakes. *Toxicon*. **21**: 279-284.

Moura-da-Silva, A.M., Paine, M.J., Diniz, M.R., Theakston, R.D. and Crampton, J.M. (1995) The molecular cloning of a phospholipase A2 from *Bothrops jararacussu* snake venom: evolution of venom group II phospholipase A2's may imply gene duplications. *J Mol Evol*. **41**: 174-179.

Neumann, D., Barchan, D., Horowitz, M., Kochva, E. and Fuchs, S. (1989) Snake acetylcholine receptor: cloning of the domain containing the four extracellular cysteines of the alpha subunit. *Proc Natl Acad Sci U S A*. **86**: 7255-7259.

Nicolaes, G.A., Villoutreix, B.O. and Dahlback, B. (2000) Mutations in a potential phospholipid binding loop in the C2 domain of factor V affecting the assembly of the prothrombinase complex. *Blood Coagul Fibrinolysis*. **11**: 89-100.

Niki, I., Yokokura, H., Sudo, T., Kato, M. and Hidaka, H. (1996) Ca^{2+} signaling and intracellular Ca^{2+}binding proteins. *J Biochem (Tokyo)*. **120**: 685-698.

Nobile, M., Noceti, F., Prestipino, G. and Possani, L.D. (1996) Helothermine, a lizard venom toxin, inhibits calcium current in cerebellar granules. *Exp Brain Res*. **110**: 15-20.

Obara, K., Fuse, N., Tsuchiya, T., Nonomura, Y., Menez, A. and Tamiya, T. (1989) Sequence analysis of a cDNA encoding a erabutoxin b from the sea-snake *Laticauda semifasciata*. *Nucleic Acids Res*. **17**: 10490.

Ogawa, T., Oda, N., Nakashima, K., Sasaki, H., Hattori, M., Sakaki, Y*., et al* (1992) Unusually high conservation of untranslated sequences in cDNAs for *Trimeresurus flavoviridis* phospholipase A2 isozymes. *Proc Natl Acad Sci U S A*. **89**: 8557-8561.

Ohana, B., Fraenkel, Y., Navon, G. and Gershoni, J.M. (1991) Molecular dissection of cholinergic binding sites: how do snakes escape the effect of their own toxins? *Biochem Biophys Res Commun*. **179**: 648-654.

Oikawa, S., Imai, M., Ueno, A., Tanaka, S., Noguchi, T., Nakazato, H*., et al* (1984) Cloning and sequence analysis of cDNA encoding a precursor for human atrial natriuretic polypeptide. *Nature*. **309**: 724-726.

Pan, S., Gulati, R., Mueske, C.S., Witt, T.A., Lerman, A., Burnett, J.C., Jr. and Simari, R.D. (2004) Gene transfer of a novel vasoactive natriuretic peptide stimulates cGMP and lowers blood pressure in mice. *Am J Physiol Heart Circ Physiol*. **286**: H2213-2218.

Pearson, J.A., Tyler, M.I., Retson, K.V. and Howden, M.E. (1993) Studies on the subunit structure of textilotoxin, a potent presynaptic neurotoxin from the venom of the Australian common brown snake (*Pseudonaja textilis*). 3. The complete amino-acid sequences of all the subunits. *Biochim Biophys Acta*. **1161**: 223-229.

Peng, L.S., Zhong, X.F., Huang, Y.S., Zhang, Y., Zheng, S.L., Wei, J.W*., et al* (2003) Molecular cloning, expression and characterization of three short chain alpha-neurotoxins from the venom of sea snake--*Hydrophiinae Hydrophis cyanocinctus* Daudin. *Toxicon*. **42**: 753-761.

Persson, E., Hogg, P.J. and Stenflo, J. (1993) Effects of Ca^{2+} binding on the protease module of factor Xa and its interaction with factor Va. Evidence for two Gla-independent $Ca(^{2+})$-binding sites in factor Xa. *J Biol Chem*. **268**: 22531-22539.

Phui Yee, J.S., Nanling, G., Afifiyan, F., Donghui, M., Siew Lay, P., Armugam, A. and Jeyaseelan, K. (2004) Snake postsynaptic neurotoxins: gene structure, phylogeny and applications in research and therapy. *Biochimie*. **86**: 137-149.

Pillet, L., Tremeau, O., Ducancel, F., Drevet, P., Zinn-Justin, S., Pinkasfeld, S.*, et al* (1993) Genetic engineering of snake toxins. Role of invariant residues in the structural and functional properties of a curaremimetic toxin, as probed by site-directed mutagenesis. *J Biol Chem.* **268**: 909-916.

Ponraj, D. and Gopalakrishnakone, P. (1995) Morphological changes induced by a generalized myotoxin (myoglobinuria-inducing toxin) from the venom of *Pseudechis australis* (king brown snake) in skeletal muscle and kidney of mice. *Toxicon.* **33**: 1453-1467.

Possani, L.D., Mochca-Morales, J., Amezcua, J., Martin, B.M., Prestipino, G. and Nobile, M. (1992a) Anionic currents of chick sensory neurons are affected by a phospholipase A2 purified from the venom of the taipan snake. *Biochim Biophys Acta.* **1134**: 210-216.

Possani, L.D., Martin, B.M., Yatani, A., Mochca-Morales, J., Zamudio, F.Z., Gurrola, G.B. and Brown, A.M. (1992b) Isolation and physiological characterization of taicatoxin, a complex toxin with specific effects on calcium channels. *Toxicon.* **30**: 1343-1364.

Ramasamy, S., Fry, B.G. and Hodgson, W.C. (2005) Neurotoxic effects of venoms from seven species of Australasian black snakes (*Pseudechis*): efficacy of black and tiger snake antivenoms. *Clin Exp Pharmacol Physiol.* **32**: 7-12.

Rao, V.S. and Kini, R.M. (2002) Pseutarin C, a prothrombin activator from *Pseudonaja textilis* venom: its structural and functional similarity to mammalian coagulation factor Xa-Va complex. *Thromb Haemost.* **88**: 611-619.

Rao, V.S., Joseph, J.S. and Kini, R.M. (2003a) Group D prothrombin activators from snake venom are structural homologues of mammalian blood coagulation factor Xa. *Biochem J.* **369**: 635-642.

Rao, V.S., Swarup, S. and Kini, R.M. (2003b) The nonenzymatic subunit of pseutarin C, a prothrombin activator from eastern brown snake (*Pseudonaja textilis*) venom, shows structural similarity to mammalian coagulation factor V. *Blood.* **1**: 1.

Rao, V.S., Swarup, S. and Kini, R. (2004) The catalytic subunit of pseutarin C, a group C prothrombin activator from the venom of *Pseudonaja textilis*, is structurally similar to mammalian blood coagulation factor Xa. *Thromb Haemost.* **92**: 509-521.

Reid, H.A. and Chan, K.E. (1968) The paradox in therapeutic defibrination. *Lancet.* **1**: 485-486.

Richards, A.M., Lainchbury, J.G., Nicholls, M.G., Cameron, A.V. and Yandle, T.G. (2002) Dendroaspis natriuretic peptide: endogenous or dubious? *Lancet.* **359**: 5-6.

Roth, D.A., Rehemtulla, A., Kaufman, R.J., Walsh, C.T., Furie, B. and Furie, B.C. (1993) Expression of bovine vitamin K-dependent carboxylase activity in baculovirus-infected insect cells. *Proc Natl Acad Sci U S A.* **90**: 8372-8376.

Rudolph, A.E., Mullane, M.P., Porche-Sorbet, R. and Miletich, J.P. (1997) Expression, purification, and characterization of recombinant human factor X. *Protein Expr Purif.* **10**: 373-378.

Schmidt, J.J. and Middlebrook, J.L. (1989) Purification, sequencing and characterization of pseudexin phospholipases A2 from *Pseudechis porphyriacus* (Australian red-bellied black snake). *Toxicon*. **27**: 805-818.

Schweitz, H., Vigne, P., Moinier, D., Frelin, C. and Lazdunski, M. (1992) A new member of the natriuretic peptide family is present in the venom of the green mamba (*Dendroaspis angusticeps*). *J Biol Chem*. **267**: 13928-13932.

Servent, D., Mourier, G., Antil, S. and Menez, A. (1998) How do snake curaremimetic toxins discriminate between nicotinic acetylcholine receptor subtypes. *Toxicol Lett*. **102-103**: 199-203.

Servent, D., Winckler-Dietrich, V., Hu, H.Y., Kessler, P., Drevet, P., Bertrand, D. and Menez, A. (1997) Only snake curaremimetic toxins with a fifth disulfide bond have high affinity for the neuronal alpha7 nicotinic receptor. *J Biol Chem*. **272**: 24279-24286.

Shea, G.M. (1999) The distribution and identification of dangerously venomous Australian terrestrial snakes. *Aust Vet J*. **77**: 791-798.

Sheumack, D.D., Spence, I., Tyler, M.I. and Howden, M.E. (1990) The complete amino acid sequence of a post-synaptic neurotoxin isolated from the venom of the Australian death adder snake *Acanthophis antarcticus*. *Comp Biochem Physiol B*. **95**: 45-50.

Singh, S.B., Armugam, A., Kini, R.M. and Jeyaseelan, K. (2000) Phospholipase A(2) with platelet aggregation inhibitor activity from *Austrelaps superbus* venom: protein purification and cDNA cloning. *Arch Biochem Biophys*. **375**: 289-303.

Slowinski, J.B., Knight, A. and Rooney, A.P. (1997) Inferring species trees from gene trees: a phylogenetic analysis of the *Elapidae* (*Serpentes*) based on the amino acid sequences of venom proteins. *Mol Phylogenet Evol*. **8**: 349-362.

Smith, H.M., Smith, R.B. and Sawn, H.L. (1977) A summary of snake classification (*Reptilia: Serpentes*). *J. Herpetol*. **11**: 115-121.

Speijer, H., Govers-Riemslag, J.W., Zwaal, R.F. and Rosing, J. (1986) Prothrombin activation by an activator from the venom of *Oxyuranus scutellatus* (Taipan snake). *J Biol Chem*. **261**: 13258-13267.

Stabeli, R.G., Marcussi, S., Carlos, G.B., Pietro, R.C., Selistre-de-Araujo, H.S., Giglio, J.R., *et al* (2004) Platelet aggregation and antibacterial effects of an l-amino acid oxidase purified from *Bothrops alternatus* snake venom. *Bioorg Med Chem*. **12**: 2881-2886.

Steen, M. (2002) Factor Va-factor Xa interactions: molecular sites involved in enzyme:cofactor assembly. *Scand J Clin Lab Invest Suppl*. **237**: 5-11.

Stiles, B.G., Sexton, F.W. and Weinstein, S.A. (1991) Antibacterial effects of different snake venoms: purification and characterization of antibacterial proteins from *Pseudechis australis* (Australian king brown or mulga snake) venom. *Toxicon*. **29**: 1129-1141.

Stocker, K. and Barlow, G.H. (1976) The coagulant enzyme from *Bothrops atrox* venom (batroxobin). *Methods Enzymol*. **45**: 214-223.

Stoupakis, G. and Klapholz, M. (2003) Natriuretic peptides: biochemistry, physiology, and therapeutic role in heart failure. *Heart Dis*. **5**: 215-223.

Stroud, R.M., McCarthy, M.P. and Shuster, M. (1990) Nicotinic acetylcholine receptor superfamily of ligand-gated ion channels. *Biochemistry*. **29**: 11009-11023.

Su, M.J., Coulter, A.R., Sutherland, S.K. and Chang, C.C. (1983) The presynaptic neuromuscular blocking effect and phospholipase A2 activity of textilotoxin, a potent toxin isolated from the venom of the Australian brown snake, *Pseudonaja textilis*. *Toxicon*. **21**: 143-151.

Sudoh, T., Kangawa, K., Minamino, N. and Matsuo, H. (1988) A new natriuretic peptide in porcine brain. *Nature*. **332**: 78-81.

Sutherland, S.K. (1983a) *Australian animal toxins*. Melbourne: Oxford University Press.

Sutherland, S.K. (1983b) P. australis*, the Mulga or King Brown snake*. Melbourne: Melbourne University Press.

Sutherland, S.K. and Leonard, R.L. (1995) Snakebite deaths in Australia 1992-1994 and a management update. *Med J Aust*. **163**: 616-618.

Sutherland, S.K. and Tibballs, J. (2001) *Australian Animal Toxins. The Creatures, Their Toxins and Care of the Poisoned Patient*. New York: Oxford University Press.

Takasaki, C. (1989) Amino acid sequence of a long-chain neurotoxin homologue, Pa ID, from the venom of an Australian elapid snake, *Pseudechis australis*. *J Biochem (Tokyo)*. **106**: 11-16.

Takasaki, C. and Tamiya, N. (1985) Isolation and amino acid sequence of a short-chain neurotoxin from an Australian elapid snake, *Pseudechis australis*. *Biochem J*. **232**: 367-371.

Takasaki, C., Yutani, F. and Kajiyashiki, T. (1990) Amino acid sequences of eight phospholipases A2 from the venom of Australian king brown snake, *Pseudechis australis*. *Toxicon*. **28**: 329-339.

Takatsuka, H., Sakurai, Y., Yoshioka, A., Kokubo, T., Usami, Y., Suzuki, M.*, et al* (2001) Molecular characterization of L-amino acid oxidase from *Agkistrodon halys blomhoffii* with special reference to platelet aggregation. *Biochim Biophys Acta*. **1544**: 267-277.

Tamiya, T., Lamouroux, A., Julien, J.F., Grima, B., Mallet, J., Fromageot, P. and Menez, A. (1985) Cloning and sequence analysis of the cDNA encoding a snake neurotoxin precursor. *Biochimie*. **67**: 185-189.

Tan, N.H. and Ponnudurai, G. (1990) A comparative study of the biological properties of Australian elapid venoms. *Comp Biochem Physiol C*. **97**: 99-106.

Tan, N.H., Ponnudurai, G. and Mirtschin, P.J. (1993a) A comparative study of the biological properties of venoms from juvenile and adult inland taipan (*Oxyuranus microlepidotus*) snake venoms. *Toxicon*. **31**: 363-367.

Tan, N.H., Ponnudurai, G. and Mirtschin, P.J. (1993b) A comparative study on the biological properties of venoms from juvenile and adult common tiger snake (*Notechis scutatus*) venoms. *Comp Biochem Physiol B*. **106**: 651-654.

Tans, G., Govers-Riemslag, J.W., van Rijn, J.L. and Rosing, J. (1985) Purification and properties of a prothrombin activator from the venom of *Notechis scutatus scutatus*. *J Biol Chem*. **260**: 9366-9372.

Thibault, G., Grove, K.L. and Deschepper, C.F. (1995) Reduced affinity of iodinated forms of Tyr0 C-type natriuretic peptide for rat natriuretic peptide receptor B. *Mol Pharmacol*. **48**: 1046-1053.

Thomas, R.G. and Pough, F.H. (1979) The effect of rattlesnake venom on digestion of prey. *Toxicon*. **17**: 221-228.

Thompson, D.F., Letassy, N.A. and Thompson, G.D. (1988) Fibrin glue: a review of its preparation, efficacy, and adverse effects as a topical hemostat. *Drug Intell Clin Pharm*. **22**: 946-952.

Thwin, M.M. and Gopalakrishnakone, P. (1998) Snake envenomation and protective natural endogenous proteins: a mini review of the recent developments (1991-1997). *Toxicon*. **36**: 1471-1482.

Torres, A.M., Wong, H.Y., Desai, M., Moochhala, S., Kuchel, P.W. and Kini, R.M. (2003) Identification of a novel family of proteins in snake venoms. Purification and structural characterization of nawaprin from *Naja nigricollis* snake venom. *J Biol Chem*. **278**: 40097-40104.

Toto, R.D., Rinner, S. and Ram, C.V. (2004) ACE inhibitors and target organ protection. An expanded role for these antihypertensive agents? *Postgrad Med*. **116**: 11-16, 19-22, 48.

Tracy, P.B., Eide, L.L. and Mann, K.G. (1985) Human prothrombinase complex assembly and function on isolated peripheral blood cell populations. *J Biol Chem*. **260**: 2119-2124.

Tremeau, O., Lemaire, C., Drevet, P., Pinkasfeld, S., Ducancel, F., Boulain, J.C. and Menez, A. (1995) Genetic engineering of snake toxins. The functional site of Erabutoxin a, as delineated by site-directed mutagenesis, includes variant residues. *J Biol Chem*. **270**: 9362-9369.

Tsetlin, V. (1999) Snake venom alpha-neurotoxins and other 'three-finger' proteins. *Eur J Biochem*. **264**: 281-286.

Tu, A.T., Hong, B. and Solie, T.N. (1971) Characterization and chemical modifications of toxins isolated from the venoms of the sea snake, *Laticauda semifasciata*, from Philippines. *Biochemistry*. **10**: 1295-1304.

Tyler, M.I., Spence, I., Barnett, D. and Howden, M.E. (1987) Pseudonajatoxin b: unusual amino acid sequence of a lethal neurotoxin from the venom of the Australian common brown snake, *Pseudonaja textilis*. *Eur J Biochem*. **166**: 139-143.

van den Bosch, H., Postema, N.M., de Haas, G.H. and van Deenen, L.L. (1965) On the positional specificity of phospholipase A from pancreas. *Biochim Biophys Acta*. **98**: 657-659.

Wakitani, K., Oshima, T., Loewy, A.D., Holmberg, S.W., Cole, B.R., Adams, S.P.*, et al* (1985) Comparative vascular pharmacology of the atriopeptins. *Circ Res*. **56**: 621-627.

Walker, F.J., Owen, W.G. and Esmon, C.T. (1980) Characterization of the prothrombin activator from the venom of *Oxyuranus scutellatus scutellatus* (taipan venom). *Biochemistry*. **19**: 1020-1023.

Walkinshaw, M.D., Saenger, W. and Maelicke, A. (1980) Three-dimensional structure of the "long" neurotoxin from cobra venom. *Proc Natl Acad Sci U S A*. **77**: 2400-2404.

Wang, Y., Jing, L. and Xu, K. (2002) A unique approach for high level expression and production of a recombinant cobra neurotoxin in *Escherichia coli*. *J Biotechnol*. **94**: 235-244.

Watanabe, T.X., Noda, Y., Chino, N., Nishiuchi, Y., Kimura, T., Sakakibara, S. and Imai, M. (1988) Structure-activity relationships of alpha-human atrial natriuretic peptide. *Eur J Pharmacol*. **147**: 49-57.

Weinstein, S.A., Williams, V. and White, J. (2001) Preliminary characteristics of the prothrombin converting enzyme from venom of Stephen's banded snake (*Hoplocephalus stephensii*). *Toxicon*. **39**: 1937-1939.

Weinstein, S.A., Bell, R.C., Brown, C.R., 2nd, Fletcher, J.E. and Smith, L.A. (1992) Pathologic changes induced by phospholipase A2 isolated from the venom of Collett's snake, *Pseudechis colletti*: a light and electron microscopic study. *Toxicon*. **30**: 171-185.

White, J. (1998) Envenoming and antivenom use in Australia. *Toxicon*. **36**: 1483-1492.

Williams, V. and White, J. (1992) Variation in the composition of the venom from a single specimen of *Pseudonaja textilis* (common brown snake) over one year. *Toxicon*. **30**: 202-206.

Wuster, W., Dumbrell, A.J., Hay, C., Pook, C.E., Williams, D.J. and Fry, B.G. (2005) Snakes across the Strait: trans-Torresian phylogeographic relationships in three genera of Australasian snakes (*Serpentes*: *Elapidae*: *Acanthophis*, *Oxyuranus*, and *Pseudechis*). *Mol Phylogenet Evol*. **34**: 1-14.

Xu, M.Q. and Evans, T.C., Jr. (2001) Intein-mediated ligation and cyclization of expressed proteins. *Methods*. **24**: 257-277.

Yamada, D., Sekiya, F. and Morita, T. (1996) Isolation and characterization of carinactivase, a novel prothrombin activator in *Echis carinatus* venom with a unique catalytic mechanism. *J Biol Chem*. **271**: 5200-5207.

Yamazaki, Y., Brown, R.L. and Morita, T. (2002) Purification and cloning of toxins from elapid venoms that target cyclic nucleotide-gated ion channels. *Biochemistry*. **41**: 11331-11337.

Yuan, Y., Jackson, S.P., Mitchell, C.A. and Salem, H.H. (1993) Purification and characterisation of a snake venom phospholipase A2: a potent inhibitor of platelet aggregation. *Thromb Res*. **70**: 471-481.

Zamudio, F., Wolf, K.M., Martin, B.M., Possani, L.D. and Chiappinelli, V.A. (1996) Two novel alpha-neurotoxins isolated from the taipan snake, *Oxyuranus scutellatus*, exhibit

reduced affinity for nicotinic acetylcholine receptors in brain and skeletal muscle. *Biochemistry*. **35**: 7910-7916.